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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this award was to study the transcriptional regulation of the BRCA2 breast cancer predisposition gene with the goal of identifying agents capable of modulating BRCA2 expression. In this project we aimed to test the effect of a variety of transcription factors, hormones, and environmental agents on BRCA2 expression and to define regions within the promoter that are responsive to these and other agents. In the course of this study we have determined that the USF binding site in the minimal promoter regulates basal expression of BRCA2. In addition we have demonstrated that the NFkB transcription factor can induce the BRCA2 promoter. In contrast, we found that adriamycin (ADR) treatment down-regulates the BRCA2 promoter 10 fold in a p53 dependent manner and that this effect is associated with inhibition of USF binding to the promoter. ADR and wildtype p53 also reduce BRCA2 protein levels but do not influence the rate of BRCA2 degradation. The promoter studies have also determined that mitomycin C but not other DNA damaging agents influences the BRCA2 promoter. No other agents were found to have a significant effect on promoter activity suggesting that BRCA2 transcription is tightly regulated.

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Fergus J. Couch 12/31/01

PI - Signature Date

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INTRODUCTION

The purpose of this award was to study the transcriptional regulation of the BRCA2 breast cancer predisposition gene with the goal of identifying agents capable of modulating BRCA2 expression. In this project we aimed to test the effect of a variety of transcription factors, hormones, and environmental agents on BRCA2 expression and to define regions within the promoter that are responsive to these and other agents. By identifying agents that regulate the expression of BRCA2 it may be possible to design methods to induce or repress the expression of this tumor suppressor gene leading to enhanced DNA repair and/or apoptosis. Thus, agents that regulate BRCA2 expression may eventually be useful as forms of therapy for breast cancer.

BODY

In this section we will describe the research that has been completed and then relate it to the specific tasks for the project.

Identification of mediators of BRCA2 basal transcription

In the first annual report for this project in February 1999, I described a series of reporter assays that were used to demonstrate that the BRCA2 reporter was not regulated by BRCA2, BRCA1, p21, p27, E2F, or SV40-T. However, a 900bp region surrounding the transcription start site of the promoter that is essential for basal activity of the promoter was identified using a series of promoter deletion constructs in luciferase promoter activity assays. In addition, mutation screening of high risk breast cancer patients for promoter mutations failed to identify any disease associated variants.

At this point a revised statement of work that focused on identifying agents and cis-elements within the promoter that regulated promoter activity. In August 1999, the 2nd annual report was filed. The mapping of an important regulatory site in the promoter between positions –58 and –18 was reported. Deletion of this site resulted in 20 fold down-regulation of the promoter. Further mapping of the site with promoter deletion constructs and point mutations implicated a tandemly repeated 9bp element located between –33 and –14 in regulation of basal transcription. This element was predicted to bind the USF transcription factor. This work directly addressed the aims proposed in Tasks 1, 4, 5, 6, and 7.

In order to identify the specific cis-acting elements from the -33 to -14 region and the specific transcription factors that bound to these elements, electrophoretic mobility shift assays were performed using oligonucleotide probes containing either wildtype sequence for the repeat region (-34 to -14) or mutations of either and both of the repeat sequences. A protein complex was found to bind the -24 to –14 site, and this complex was super shifted by antibody against USF (kindly provided by Michele Sawadogo) but was not affected by antibodies against c-myc (Santa Cruz Biotechnology) or ATF2 (Santa Cruz

Biotechnology). This suggested that USF is required for basal activity of the BRCA2 promoter. This work directly addressed the aims in Tasks 7 and 9.

In the next annual report in August 2000, a series of experiments verifying the role of USF in regulation of BRCA2 promoter activity were outlined. Specifically, ectopic expression of USF1 or USF2 in Saos-2 and HMEC cells resulted in induction of the BRCA2 promoter as measured by a luciferase reporter construct and by increased levels of wildtype BRCA2 mRNA. These data and those presented in the1999 report are reported in the manuscript attached in Appendix 1 (Wu et al., 2000).

Induction of the BRCA2 promoter by NFκB.
In the August 2000 report systematic mapping of other transcription factor binding sites within the BRCA2 promoter which contribute to regulation of the promoter was described. Initially, a -144 to -58 region was shown to induce basal transcription 3-fold. Sequence analysis of this region identified several putative transcription factor binding sites including an NFκB consensus binding site located at position -116 to –107. Expression of NFκB subunits in cells with the BRCA2 promoter resulted 9 to 16-fold induction of promoter activity. In subsequent experiments it was demonstrated that 1) NFkB dependent activation of the promoter requires the NFkB consensus binding site, 2) the p50 NFkB subunit binds to the NFkB consensus binding site in the promoter, 3) overexpression of NFkB subunits leads to in vivo induction of BRCA2, and 4) dominant negative and wildtype IκBα inhibit NFκB dependent induction of BRCA2. The studies demonstrating these effects are outlined in detail in the manuscript attached in Appendix 1 (Wu et al., 2000). These data clearly demonstrate that the NFκB transcription factor can induce BRCA2 expression by binding to the BRCA2 promoter. Thus, these experiments address the aims stated in Task 2, 3, 7, 8, 9, and 10.

The effect of pharmacological and physiological agents on BRCA2 promoter activity. As outlined in Task 2 and 3, we proposed to study the ability of various agents to regulate BRCA2 expression. In August 2000 it was reported that adriamycin, estrogen, serum starvation, forskolin, and TNFα altered BRCA2 promoter activity, while UV-irradiation, gamma-irradiation, camptothecin, taxol, INFγ and vincristine appeared to have little effect. In the last year we have evaluated the effects of additional agents and proteins on BRCA2 promoter activity using luciferase assays and northern blots.

We have evaluated the effect of the estrogen analogues TCDD and Quercetin on the promoter because the minimal promoter contains two xenobiotic response elements (XRE sites) that may bind or be activated by these agents. A total of 1 X 10^5 MCF7 and T47D breast cancer cells were plated in each well of a 6 well plate. After 24hrs these cells were exposed to 2, 5, or 10 nM TCDD, or 5, 10, 25, 50, and 100 μM Quercetin. Neither agent altered promoter activity by greater than 2 fold suggesting that these agents have limited ability to regulate BRCA2 transcription. Northern blots again showed only minor increases in BRCA2 mRNA.

In addition, we followed up on the observation that certain hormones regulate BRCA2 expression. As reported previously, treatment of cells with 1 μM estrogen induced a two fold increase in promoter activity. However, further increases in estrogen failed to induce proportional increases in promoter activity, suggesting that estrogen and the estrogen receptor were not directly affecting the BRCA2 promoter. Subsequently a series of BRCA2 promoter deletion constructs were treated with estrogen in an effort to identify an estrogen receptor responsive site in the promoter. No alterations in promoter activity were detected suggesting that the estrogen receptor does not bind to the promoter and that the estrogen effect on the promoter is a result of general effects on cell growth. We also evaluated the effect of androgens and the androgen receptor on the BRCA2 promoter. Androgen receptor null DU145 cells were transfected with an androgen receptor expression construct and were treated with 1 μM R1881 artificial androgen. Again only a two fold effect was detected. Various mutants of the androgen receptor or of the BRCA2 promoter did not alter this effect suggesting that the androgen receptor does not directly effect the promoter. These studies directly address the aims of Tasks 2, 3, and 8.

Characterization of an inhibitory region in the BRCA2 promoter

In the August 2000 report we described a 200bp inhibitory region between positions –500 and –700bp in the BRCA2 promoter. In the last year we have further mapped this repression site to a 56bp region using other deletion constructs. In an effort to better map this inhibitory region we used linker scanning to mutate a series of overlapping 6bp sites across this region. Luciferase assays were then performed with these mutant promoter constructs. Promoter activity increased 2 fold in the presence of 7 different mutations suggesting that the repression region is 46bp in size. In addition mutation of an Ets transcription factor binding site reduced activity 3 fold. This suggests that this region of the promoter binds both positively and negatively regulating transcription factors in a complex fashion. Interestingly we have also determined that this ets site cooperates with two other ets sites located in the minimal promoter to regulate basal activity of the BRCA2 promoter. This study directly addresses Tasks 2, 7, and 8. We have not pursued either of these observations.

The mechanism of BRCA2 inhibition by adriamycin.

In the August 2000 report for this study we outlined evidence suggesting that adriamycin (ADR) downregulates the BRCA2 promoter. In these experiments we determined that 5 μM ADR reduced promoter activity 10 fold in MCF7 cells, and that the effect was entirely dependent on p53. However, a number of questions remained. In the last year we have followed up on this study in order to confirm our hypothesis that ADR specifically inhibits BRCA2 expression though the BRCA2 promoter.

All of our prior experiments were performed with 5 μM ADR. However, this is a very high dose of drug. Therefore, we repeated the experiments using 0.7 μM ADR. The results were essentially identical to those obtained with 5 μM ADR. We also repeated all of the experiments in several different cell lines to ensure that the ADR effect was not specific to MCF7 cells. Specifically, we used HCT116 colon carcinoma cells, and U2OS osteosarcoma cells, both of which are wildtype for p53. As before all results matched the results from the MCF7 cells. In order to verify that the ADR associated repression of the promoter is dependent on wildtype p53 we repeated the experiments in a series of p53 null or p53 mutant cell lines. Specifically, we used Saos2 osteosarcoma cells, HCT116 p53-/- colon carcinoma cells, T47D breast adenocarcimona cells, and MCF7 cells stably expressing the HPV E6 gene that leads to rapid degradation of p53. None of these cells showed any reduction in promoter activity in response to ADR treatment, while transfection of wildtype p53 into the cells led to a reduction in activity. To verify that the ADR and p53 dependent effects on the promoter in the various cell lines results from inhibition of USF-1 binding to the USF cis element in the minimal promoter we performed gel shift and supershift assays. Gel shift assays using protein lysates from MCF7, U2OS, and HCT116 cells detected a reduction in the amount of DNA/protein complex formation when cells were treated with ADR. Supeshift assays verified that USF-1 was present in this complex. In contrast, p53 null or mutant cells showed no change in complex formation in response to ADR.

While these data strongly suggest an effect of ADR and p53 on the promoter, it did not establish whether ADR and p53 dependent repression of the promoter had any effect on BRCA2 protein levels in cells. To evaluate this possibility, each of the cell types mentioned above were treated with ADR and western blots of cell lysates were performed using the Ab-1 antibody from Oncogene Research Inc. As expected, cells containing wildtype p53 showed a systematic reduction in BRCA2 protein levels in parallel with BRCA2 promoter repression, while no effects on protein levels were observed in p53 mutant or null cells. Next we evaluated whether the effects on protein levels were due to reduced gene expression because of promoter repression or due to altered rates of BRCA2 protein degradation. Cells were treated with cyclohexamide alone to blot protein synthesis or with cyclohexamide and 5 μM ADR for 0, 1,2,4,6,8, and10 hours and protein lysates from each time point were western blotted for BRCA2 with the Ab-1 antibody. Results showed that the rate of BRCA2 protein degradation was not altered by treatment with ADR. Taken together, these data strongly suggest that ADR specifically represses BRCA2 promoter activity in a p53 dependent fashion but has no effect on BRCA2 protein turnover. A manuscript describing these studies is in preparation. A preliminary draft of this manuscript is included in Appendix 2.

Interestingly, UV-irradiation, gamma-irradiation, camptothecin, and taxol are all known to induce p53 as a result of their DNA damaging activity. However, none of these agents had any effect on the BRCA2 promoter. In contrast, both actinomycin D and mitomycin C induced p53 and repressed the BRCA2 promoter through the USF1 site. These agents intercalate into the DNA similarly to adriamycin. Thus, the intercalating agents must induce a 2nd factor in addition to p53 that leads to inhibition of the BRCA2 promoter during S-phase of the cell cycle. We have not attempted to identify this 2nd factor. The experiments described above directly address the aims stated in Task 2, 3, 7, 8, 9, and 10.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that UV irradiation, γ-irradiation, camptothecin, taxol, vincristine, and INFγ have no effect of the BRCA2 promoter.
- Observed that Forskolin, TNFα, estrogen, and serum starvation alter BRCA2 promoter function

- Determined that the estrogen analogues quercetin and TCDD have no effect on the BRCA2 promoter
- Showed that the androgen receptor and the estrogen receptor have no direct effect on the BRCA2 promoter
- Showed that mitomycin C and actinomycin D repress BRCA2 promoter activity
- Determined that adriamycin significantly downregulates the BRCA2 promoter
- Demonstrated that the USF cis element regulates basla activity of the BRCA2 promoter
- Determined that NFkB transcription factor can upregulate BRCA2 expression
- Demonstrated that wildtype p53 is required for ADR dependent downregulation of BRCA2 expression
- Identified the USF-1 binding site as the mediator of the adriamycin and p53 effect on the BRCA2 promoter
- Determined that ADR treatment and wildtype p53 expression results in reductions in BRCA2 protein levels
- Demonstrated that ADR does not influence the rate of BRCA2 protein degradation

REPORTABLE OUTCOMES

Wu K, Jiang S-W, Thangaraju M, Wu G, **Couch FJ**. Induction of the BRCA2 Promoter by Nuclear Factor-κβ. J. Biol. Chem 275(45):35548-35556, 2000.

CONCLUSIONS

In summary, we have shown that the BRCA2 promoter can be downregulated by both adriamycin and p53. Adriamycin damages DNA, while BRCA2 has been associated with DNA damage repair. By repressing BRCA2 expression, adriamycin can prevent DNA damage repair while causing DNA damage. In addition, adriamycin induces p53 expression which can activate apoptotic signaling pathways in response to DNA damage. Thus, the combination of DNA damage, induction of p53 dependent apoptosis, and inhibition of BRCA2 dependent DNA repair by adriamycin suggests that this drug is particularly well suited as a chemotherapeutic agent.

It is also clear from our data that the BRCA2 promoter is very tightly regulated in a cell cycle dependent manner. We were unable to identify many factors or pharmacological agents that influenced promoter activity, and those that did had very minor effects. Further evidence of tight regulation of BRCA2 signaling comes from the p53 response. Here we have shown that p53 can repress the BRCA2 promoter, while others have shown that BRCA2 represses p53 dependent transcription. This suggests that in the absence of p53 induction BRCA2 is active and downregulates p53 dependent transcription, thereby maintaining cell viability. However upon induction of p53, BRCA2 is downregulated and p53 dependent transcription is activated. Thus, BRCA2 and p53 appear to form an interactive loop.

The most surprising result in this study was the observation that DNA damage does not influence BRCA2 transcription. Because of the role of BRCA2 in DNA damage repair it was expected that DNA damage would influence the BRCA2 promoter. While p53 does appear to repress the promoter, or in fact prevent induction of the promoter, induction of p53 by DNA damaging agents is not sufficient to influence promoter activity. Thus, a 2nd as yet unknown factor seems to be involved in the process.

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Induction of the BRCA2 Promoter by Nuclear Factor-κB*

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BRCA2 is a tumor suppressor gene that has been implicated in response to DNA damage, cell cycle control, and transcription. BRCA2 has been found to be overexpressed in many breast tumors, suggesting that altered expression of the BRCA2 gene may contribute to breast tumorigenesis. To determine how BRCA2 is overexpressed in tumors, we investigated the transcriptional regulation of the BRCA2 promoter. Deletion mapping of the BRCA2 promoter identified three regions associated with 3-fold activation or repression and one upstream stimulatory factor binding site associated with 20-fold activation. Gel shift and cotransfection studies verified the role of USF in regulation of BRCA2 transcription. Analysis of the −144 to −59 region associated with 3-fold activation identified a putative NFκB binding site. Cotransfection of the p65 and p50 subunits of NFκB upregulated the BRCA2 promoter 16-fold in a luciferase reporter assay, whereas mutations in the binding site ablated the effect. Gel shift and supershift assays with anti-p65 and -p50 antibodies demonstrated that NFκB binds specifically to the NFκB site. In addition, ectopic expression of NFκB resulted in increased levels of endogeneous BRCA2 expression. Thus, NFκB and USF regulate BRCA2 expression through the BRCA2 promoter.

BRCA2 is a tumor suppressor gene associated with familial predisposition to breast and ovarian cancer (1, 2). Mutations in BRCA2 are thought to account for 20–35% of all inherited breast cancers and are associated with a 37–85% lifetime risk of developing cancer (3, 4). The great majority of disease-associated mutations in BRCA2 result in truncation of the BRCA2 protein, suggesting that loss of function of BRCA2 results in tumor susceptibility. However, the mechanisms by which the BRCA2 protein suppresses tumor cell growth are largely unknown.

The BRCA2 gene encodes a 3418-amino acid nuclear protein (2, 5), that has been implicated in the cellular response to DNA damage. BRCA2 interacts directly with RAD51, a protein involved in meiotic and mitotic recombination, DNA double-stranded break repair, and chromosome segregation (6, 7), through the BRC repeats and a C-terminal binding site. BRCA2$^{-/-}$ animals die as early embryos (8–11), and viable BRCA2$^{-/-}$ early mouse embryos are highly sensitive to γ-irradiation-induced DNA damage (9). Moreover, cells expressing mutant BRCA2 are more sensitive to methyl methanesulfonate-induced DNA damage than cells expressing wild type BRCA2 (12), and BRCA2 appears to be required for ionizing radiation-induced assembly of a RAD51 protein complex *in vivo* (13).

BRCA2 may be also involved in regulation of the cell cycle and genome instability. BRCA2 is expressed in a cell cycle-dependent manner with peak expression in the S and G_2 phases of the cell cycle. Low levels of expression are detected in G_0, G_1, and M phase (14). Cell cycle-dependent expression has recently been associated with binding of the upstream stimulatory factor (USF)[1] protein and Elf-1 transcription factor to the BRCA2 promoter (15). In addition, BRCA2 expression is elevated indirectly in response to the mitogenic activity of estrogen, which has been associated with progression of the cell cycle (16, 17). Furthermore, recent studies of BRCA2$^{-/-}$ mouse embryo fibroblasts identified extensive chromosomal rearrangement, centrosome amplification, and aneuploidy, consistent with abrogation of a mitotic checkpoint (18). Likewise, tumor cells expressing mutant BRCA2 have been shown to contain multiple chromosomal rearrangements (19). These data suggest that BRCA2 plays a key role in regulation of cell growth and proliferation in many cell types.

Several studies have attempted to define a role for BRCA2 in development of sporadic breast cancer (20–25). Loss of heterozygosity of the BRCA2 locus has been detected in over 50% of sporadic breast tumors, suggesting a role for BRCA2 in sporadic breast cancer development (20–22). However, no somatic mutations of BRCA2 have been found in sporadic breast cancers (23, 24). Also, the BRCA2 promoter is not inactivated by methylation in breast tumors (25). Although no sequence alterations have been found in the BRCA2 gene in sporadic tumors, it remains possible that BRCA2 does contribute to sporadic breast cancer development, albeit not by inactivation of the BRCA2 protein through mutagenesis and methylation. One possible mechanism of BRCA2 involvement is through deregulated expression of the BRCA2 gene. Recently, it has been shown that BRCA2 is significantly overexpressed in many sporadic breast cancers (26). It is not known whether this overexpression of BRCA2 is due to induction of the BRCA2 promoter or is a result of an increased number of cells in S phase of the cell cycle. However, when combining this observation with the known relevance of BRCA2 function to regulation of cell proliferation, it seems likely that expression of the BRCA2 gene is tightly regulated and that altered expression of BRCA2 may contribute to breast cancer development.

To begin to assess the contribution of altered expression of

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[1] The abbreviations used are: USF, upstream stimulatory factor; NFκB, nuclear factor-κB; IκBα, inhibitor of κB; PCR, polymerase chain reaction; ATF, activating transcription factor; CREB, CRE-binding protein; MLTF, major late transcription factor; bp, base pair(s); kb, kilobase(s); dn-IκBα, dominant negative IκBα 32A/36A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

BRCA2 to breast tumorigenesis, we investigated the transcriptional regulation of the BRCA2 promoter. Here we provide evidence for direct induction of the BRCA2 promoter through binding of the nuclear factor-κB (NFκB) transcription factor, and we verify the role of USF in regulation of basal activity of the promoter.

EXPERIMENTAL PROCEDURES

Cell Culture—Human breast adenocarcinoma MCF-7 cells were obtained from American Type Culture Collection, propagated in the Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (HyClone) and maintained at 37 °C with 5% CO_2. Cell culture reagents were obtained from Life Technologies, Inc.

BRCA2 Reporter Constructs—A BAC clone (B489G) containing the 5′ end of the BRCA2 gene was isolated from a BAC library (27) using a polymerase chain reaction (PCR)-generated hybridization probe consisting of bases 72–560 of the BRCA2 cDNA. B489G DNA was digested with *Sac*I and *Pst*I enzymes, and the resulting fragments were subcloned into the pGL3 basic vector containing a firefly luciferase reporter gene (Promega) and plated. Colonies containing the 5′ end of the BRCA2 gene were identified by hybridization with the 72–560-bp cDNA probe. Plasmid DNA from positive colonies was prepared and sequenced using vector specific primers. Sequences were then matched against the complete genomic sequence of this region in GenBank™. A clone with an 8-kb insert (pGL3Prom) was found to include 4.3 kb of sequence upstream of the putative BRCA2 transcription start site and 3.7 kb downstream of the transcription start site including exons 1, 2, and 3 of BRCA2. The entire 8-kb insert was then sequenced by the Molecular Biology Core of the Mayo Clinic.

Deletion Mutants of the BRCA2 Promoter—A series of deletion mutants (see Figs. 1 and 2) of the BRCA2 promoter were generated by restriction enzyme digestion with a variety of restriction enzymes followed by religation and also by direct PCR amplification. The Del-1 construct was generated by digesting the pGL3Prom construct with *Hin*dIII and *Pst*I and religating the pGL3Prom plasmid. Del-2 resulted from religation following digestion with *Mlu*I and *Pst*I. Del-9 was generated by subcloning a 1249-bp fragment of pGL3Prom, resulting from *Kpn*I and *Mlu*I digestion, into the pGL3 basic promoter. Del-2 was then digested by combinations of *Sac*I with *Nde*I, *Hin*dIII, *Eco*RI, and *Bbr*PI, and the linearized plasmids were blunt-ended with Klenow enzyme (New England BioLabs) and religated to form Del-3, Del-4, Del-5, and Del-16, respectively.

Additional deletion mutants were constructed by PCR-based strategies. PCR primers were designed containing a *Sac*I or *Kpn*I site in 5′ forward primers and a *Pst*I site in 3′ reverse primers. The 5′ forward primers used in the constructs Del-6–8, and Del-10–15 were: Del-6 (−897), 5′-TGGGTGTGGGAGCTCATGCCTGTAATCC-3′; Del-7 (−796), 5′-AAACCCCGAGCTCTACTTAAAAATGCA-3′; Del-8 (−678), 5′-GGAAGTTGCGGTGAGCTCAGATTGCG-3′; Del-10 (−515), 5′-ACGGGCTCGGAGCTCTTGAACAC-3′; Del-11 (−422), 5′-ACTAAGTGAGCTCATCCACAACC-3′; Del-12 (−310), 5′-AAGGTATTTCAGAGCTCCCAGG-3′; Del-13 (−236), 5′-GACTTGGAGCTCAGGCATAGG-3′; Del-14 (−144), 5′-TATTCGAGCTCAGATACTGACGG-3′; and Del-15 (−58), 5′-CCAGGCCTGAGCTCCGGGTG-3′. The single 3′ reverse primer was 5′-AGCCCGGGCCTGCAGGCGTGGCTAG-3′, which contains a *Pst*I site. The 3′ reverse primers used for Del-17 and Del-18 were: Del-17 (0), 5′-TTCAGAAGCTCGCTGCAGAAGCCCGCGCTGG-3′ and Del-18 (+110), 5′-TCTGTCCCCTGCAGGCTTCTCC-3′. The single 5′ forward primer was 5′-TGCGGAGCAAGGGAGCTCACACTTCATGAGC-3′, which contains a *Kpn*I site. PCR products were generated using *Pfu* DNA polymerase (Stratagene) and 30 ng of Del-2 as template DNA. PCR conditions were as follows: 1 cycle for 2 min at 95 °C; 20 cycles at 95 °C for 30 s, 55 °C for 1 min and 68 °C for 4 min; and 1 cycle at 68 °C for 10 min. The PCR products were digested either with *Sac*I and *Pst*I or with *Kpn*I and *Pst*I restriction enzymes and ligated into pGL3 basic vectors. Deletion mutants generated with PCR were sequenced using an automated DNA sequencer to monitor for PCR-associated nucleotide incorporation errors.

Point Mutants of the BRCA2 Promoter—Site-directed mutagenesis of the Del-15 construct was performed using the QuikChange site-directed mutagenesis kit (Stratagene) to prepare constructs containing mutations in predicted *cis*-elements within the promoter. Specifically, mutations were introduced into putative DNA-binding sites for the ATF, USF, MLTF, and c-Myc transcription factors (see Fig. 2). Mutations were confirmed by DNA sequencing.

Luciferase Reporter Assays—Plasmid DNA for transient transfection was isolated using the plasmid maxi kit (Qiagen). MCF-7 cells were plated at a density of 1×10^5 cells/well of 6-well plates and grown in Dulbecco's modified Eagle's medium with 10% bovine calf serum overnight prior to transfection. All transfections were carried out using Fugene-6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. A total of 2 μg of BRCA2 promoter construct and 0.1 μg of pRL-TK *Renilla* luciferase vector (Promega) with 4 μl of Fugene-6 was used for each transfection. The pRL-TK *Renilla* luciferase activity was used to control for transfection efficiency. Each transfection experiment was performed in duplicate and repeated a minimum of three times. For cotransfection experiments, cells received 0.5 μg of BRCA2 promoter construct, 0.1 μg of pRL-TK *Renilla* luciferase vector, and 0.5 μg of the indicated expression plasmids and carrier DNA. Expression plasmids included pCMV-USF, pCMV-USF-VP16, pCMV-VP16, pCMV, pcDNA3.1-p65, pcDNA3.1-p50, pcDNA3.1, pCMV-CREB, pCMV-Myc, and pCMV-Max. Firefly luciferase and *Renilla* luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Approximately 48 h after transfection, cells were washed twice with 1× phosphate-buffered saline and harvested with 600 μl of passive lysis buffer (Promega). Cell lysates were cleared by centrifugation, and 5 μl was added to 100 μl of firefly luciferase substrate, and light units were measured in a luminometer. *Renilla* luciferase activities were measured in the same tube after addition of 100 μl of Stop and Glo reagent.

Electrophoretic Mobility Shift Assays—Double strand oligonucleotides generated from the single strand oligonucleotides listed in Table I and II were used as electrophoretic mobility shift assay probes. The upper strand (sense) oligonucleotide (30 ng) was 5′ end labeled using polynucleotide kinase with [γ-^{32}P]dATP (Amersham Pharmacia Biotech). After the labeling reaction, 2-fold excess of lower strand (antisense) oligonucleotide was annealed to the upper strand. Double-stranded DNA probes were purified from the reaction mixture using a Bio-Gel P-100 column (Bio-Rad). Whole cell extracts were isolated from cultured MCF-7 cells. DNA-protein binding was performed in 0.5× Dignam buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μg/ml poly(dI-dC). Binding reactions were initiated by addition of 30,000 cpm DNA probe in TE buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA) to 5–10 μl of whole cell extracts. Electrophoresis was performed in acrylamide gels, gels were dried and exposed to film for 16–48 h.

Competition experiments were carried out in the same way as described above except that increasing amounts of double-stranded wild type oligonucleotide were mixed with 30,000 cpm of M-1 probe (see Table I) and added to the binding reaction. For optimized antibody mediated supershift experiments, increased DNA probe (60,000 cpm) and decreased whole cell extracts (5 μl) were applied. The binding reaction included 1–4 μl of antibodies against ATF2 (Santa Cruz Biotech), c-Myc (Santa Cruz Biotech) or USF-1 (kindly provided by Dr. Michele Sawadogo, M.D. Anderson Cancer Center).

DNA binding assays for NFκB were also performed using electrophoretic mobility shift assays. Whole cell extracts were prepared from MCF-7 cells 48 h after transfection with pcDNA 3.1 or NFκB p65 and p50 subunit expression constructs. Components of NFκB proteins were identified by supershift assay using antibodies against p50 and p65 (Santa Cruz Biotech).

Western Blotting—48 h after transfection with pcDNA 3.1 or p50 and p65 expression constructs, MCF-7 cells were washed with 1× phosphate-buffered saline, and cell lysates were prepared with RIPA buffer containing COMPLETE proteinase inhibitor mixture (Roche Molecular Biochemicals). Equal amounts of protein lysate from each transfection were subjected to electrophoresis, transferred to membrane, and probed with primary antibodies and alkaline phosphatase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Signals were developed by ECL detection system.

RNA Isolation and Northern Blotting—Total RNA was isolated from MCF-7 cells 48 h after transfection with pcDNA 3.1 or p50 and p65 expression constructs, or pcDNA3.1, dominant negative IκBα 32A/36A (dn-IκBα), NFκB p65, IκBα wild type, p65 with dn-IκBα, or p65 with wild type IκBα using TRIzol reagent (Life Technologies, Inc.), according to the manufacturer's instructions. Total RNA samples (20 μg/lane) for pcDNA 3.1, p50, and p65 transfected cells were resolved on 0.8% agarose-formaldehyde gels and transferred to nylon membranes. The membranes were prehybridized at 62 °C for 1 h in ExpressHyb Hybridization Solution (CLONTECH) and then hybridized for 1 h in the same solution with [α-^{32}P]ATP random labeled full-length human BRCA2 cDNA. After hybridization, the membrane were washed (three times for 15 min each time at room temperature) with 2× SSC, 0.05% SDS and washed (three times for 15 min each time at 62 °C) with 0.5× SSC, 0.1%

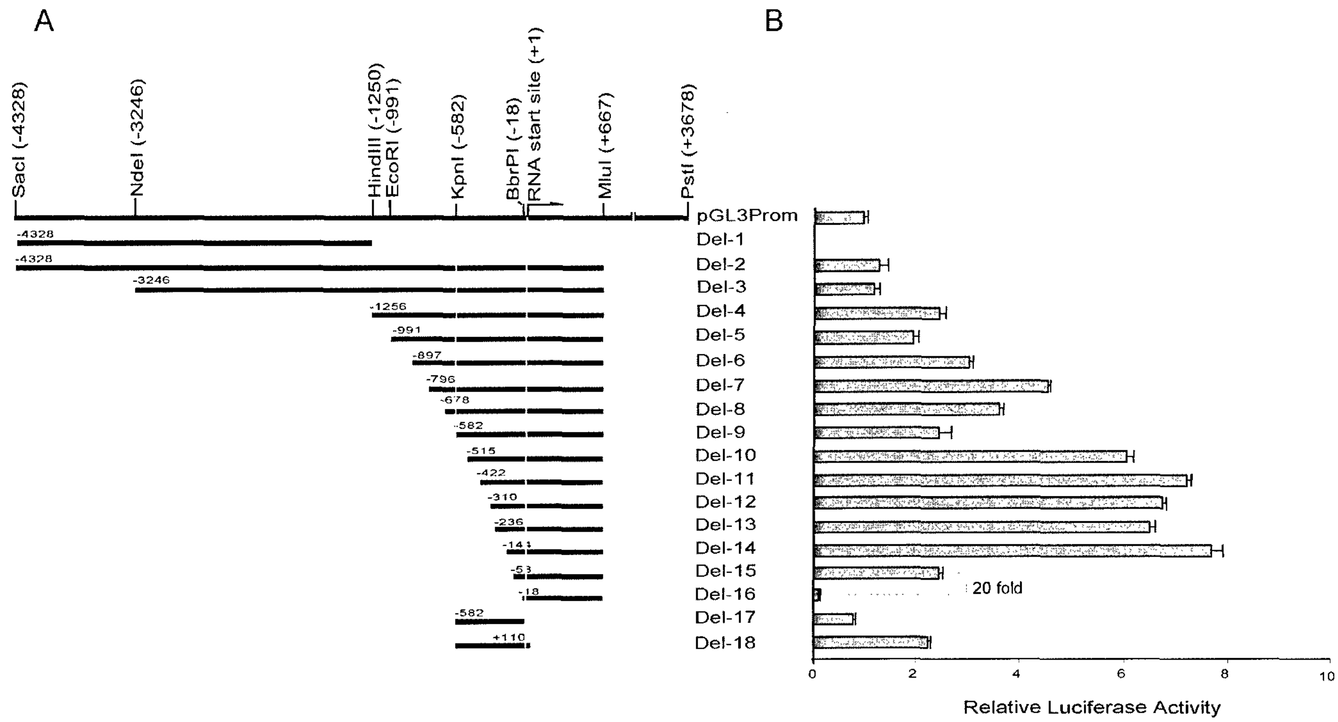

FIG. 1. **Activity profiles of human BRCA2 promoter luciferase reporter deletion constructs in MCF-7 cells.** *A*, schematic diagram of an 8-kb fragment of genomic DNA containing the BRCA2 promoter cloned into the pGL3 reporter construct (pGL3Prom). A series of deletion constructs are also shown. The position of the most proximal or distal nucleotide from the promoter region relative to the transcription start site of BRCA2 is shown for each construct. *B*, luciferase activity profiles of the BRCA2 promoter reporter constructs in MCF-7 cells. To control for transfection, efficiency cells were cotransfected with pRL-TK, and the activity associated with each construct was normalized relative to *Renilla* luciferase activity. The luciferase activity for each construct is shown relative to the wild type pGL3Prom construct.

SDS. Membranes were then exposed in a PhosphorImager. Each membrane was also hybridized as described above with a GAPDH probe for normalization of mRNA levels.

Semi-quantitative Reverse Transcription-PCR Analysis—1 μg of total RNA from cells transfected with pcDNA3.1, dn-IκBα, NFκB p65, IκBα wild type, p65 with dn-IκBα, or p65 with wild type IκBα was used for preparation of cDNA with random hexamer primers and superscript II reverse transcriptase (Life Technologies, Inc.). After treatment with DNase, 2 μl from a total of 100 μl was used for semi-quantitative PCR with BRCA2 and GAPDH PCR primers. The sequences of forward (F) and reverse (R) PCR primers were as following: BRCA2, 5′-GCAGTGAAGAATGCAGCAGA-3′ (F, within the exon 21 of human BRCA2) and 5′-CAATACGCAACTTCCACACG-3′ (R, within the exon 22 of human BRCA2); GAPDH, 5′-CAACTACATGGTTTACATGTTC-3′ (F) and 5′-GCCAGTGGACTCCACGAC-3′ (R). Each PCR amplification was performed using *Taq* DNA polymerase (Promega) with both PCR primers for BRCA2 and GAPDH under the following conditions: 1 cycle for 2 min at 94 °C; 25 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and 1 cycle at 72 °C for 10 min. The GAPDH product was used as a normalization control for the amount of cDNA in the PCR reactions. PCR products were subjected to electrophoresis using 6% polyacrylamide gels and stained by Sybr Green for 1 h. Results were analyzed with a Molecular Dynamics PhosphorImager system.

RESULTS

Identification of Regulatory Domains in the BRCA2 Promoter—To analyze transcriptional regulation of the BRCA2 gene and to define functionally important *cis*-DNA elements in the 5′-flanking region of this gene, an 8-kb region of human genomic DNA containing the BRCA2 putative promoter was isolated from BAC clone B489G and subcloned into the pGL3 basic luciferase reporter vector. The 8-kb fragment contained 4.3 kb of sequence upstream of the putative transcription start site (2) and 3.7 kb downstream of the transcription start site as far as the 3′ donor splice site of exon 3. This pGL3Prom and the pGL3 parent vector were transiently transfected into MCF-7 cells, and luciferase activity was measured after 48 h. All activities were normalized by activity measurements from cotransfected pRL-TK *Renilla* luciferase vector. The pGL3Prom construct yielded 100-fold more luciferase activity than pGL3, suggesting that the pGL3Prom construct contained the BRCA2 promoter.

To identify the minimal BRCA2 promoter, a series of deletion constructs (Fig. 1*A*) derived from pGL3Prom were generated as described above. Firefly luciferase expression was assayed following transient transfection of MCF-7 cells with these BRCA2 promoter constructs. The normalized luciferase activities for each deletion construct of the promoter relative to pGL3Prom activity are shown in Fig. 1*B*. The results indicate that the BRCA2 promoter is regulated in a complex fashion. No change in activity was detected when comparing the Del-2 construct with pGL3Prom, suggesting that the +668 to +3678 region has no influence on promoter activity. Deletion of the −4328 to −583 region caused a 2-fold increase in luciferase activity. Further deletion from −582 to −516 resulted in 2.5-fold activation of the promoter, whereas a 3-fold reduction in activity was detected following deletion of the −144 to −59 region. However, a 20-fold loss of luciferase activity was observed following deletion of a 40-bp region (−58 to −19), suggesting that the region contains *cis*-elements that are critical for positive regulation of basal transcription activity in the BRCA2 promoter.

Analysis of the Minimal BRCA2 Promoter—To more accurately map the *cis*-element within the −58 to −19 region that regulates BRCA2 basal transcription, a further series of deletions were constructed using the Del-15 construct as a template (Fig. 2*A*). Deletion of the −34 to −19 region (Del-16) resulted in a 12-fold reduction in luciferase activity in comparison to Del-19 (Fig. 2*A*). Sequence analysis of a 20-bp region from −34 to −14 was carried out in an effort to identify putative transcription factor binding sites that might regulate BRCA2 basal transcription activity. The region was found to contain a tan-

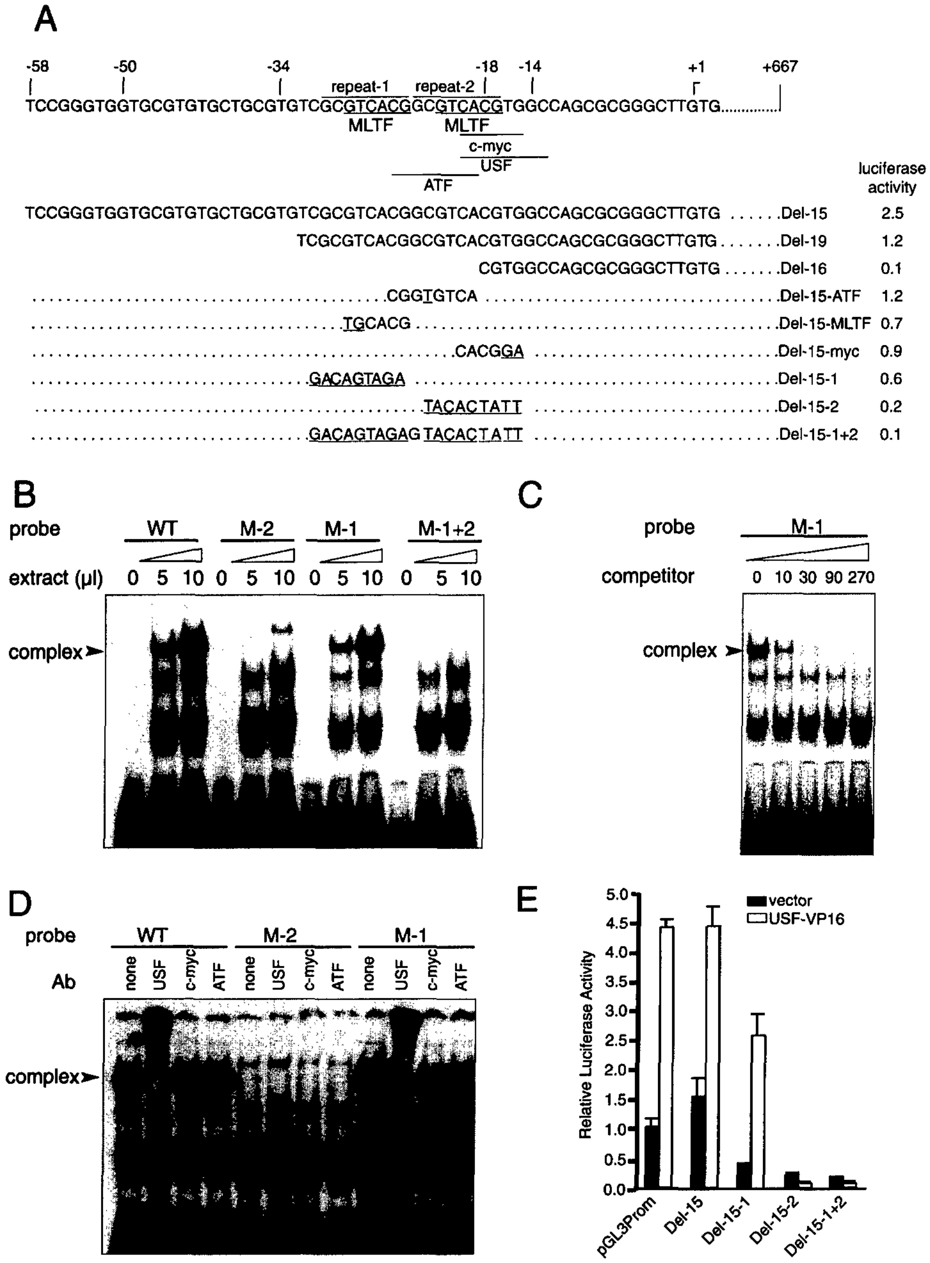

FIG. 2. **USF regulates BRCA2 basal transcription.** *A*, the minimal BRCA2 promoter. The minimal promoter contains the region between positions −58 and +3 relative to the transcription start site. A series of deletion and substitution constructs derived from Del-15 are also shown. Luciferase activities of the BRCA2 promoter reporter constructs in MCF-7 cells relative to the wild type pGL3Prom construct are indicated. The positions of a tandem repeat 8-bp sequence, and putative MLTF, ATF, USF, and c-Myc binding sites are indicated. Substituted nucleotides from each mutant construct are *underlined*. *B*, a single protein complex binds to the 20-bp repeat sequence. Electrophoretic mobility shift assays using wild type (*WT*) and mutant (*M-1* and *M-2*) oligonucleotide probes (Table I) and MCF-7 whole cell protein extracts were performed. *M-1* contains substitution mutations in the first 8-bp repeat, and *M-2* contains mutations in the second 8-bp repeat. The single protein complex is indicated. *C*, a protein complex binds specifically to the second 8-bp repeat. A competition assay was performed using increasing concentrations of unlabeled wild type oligonucleotide probe. *D*, the USF transcription factor binds to the second 8-bp repeat. Supershift assays were performed with whole cell lysates of MCF-7, anti-USF-1, c-Myc, and ATF antibodies, and wild type and mutant oligonucleotides (*WT*, *M-1*, and *M-2*). The supershifted complex containing the anti-USF antibody and the gel shift complex are indicated. *E*, USF requires a transactivating cofactor to activate the BRCA2 promoter. MCF7 cells were transfected with pGL3Prom, Del-15, Del-15–1 (substitution in the first repeat), Del-15–2 (substitution in the second repeat), or Del-15–1+2 (substitutions in both repeats) constructs along with a pCMV-USF-VP16 construct containing a USF and VP16 fusion gene or a vector control. Luciferase activities were normalized by protein concentration and are shown relative to the activity from the pGL3Prom wild type construct.

dem GCGTCACG repeat (Fig. 2*A*) that encodes several predicted transcription factor binding sites including *cis*-elements for c-Myc, USF, MLTF, and ATF transcription factors. A number of point mutations were introduced into the 16-bp repeat sequence in the Del-15 reporter construct in an effort to identify the *cis*-element, which was regulating basal transcription from the promoter. Substitution of nucleotides from repeat 1 resulted in a 4-fold loss in activity, whereas substitution of repeat 2 led to a 12-fold loss in luciferase activity (Fig. 2*A*). Thus, both 8-bp repeats appear to be involved in regulation of basal transcription. Further mutation studies eliminated the ATF, c-Myc, and MLTF binding sites from consideration and determined that BRCA2 basal transcription is predominantly regulated through the USF binding site.

USF Regulates BRCA2 Basal Transcription—The −34 to −15 region has recently been reported to be responsible for regulation of the basal activity of the BRCA2 promoter (15). The USF binding site was shown to regulate promoter activity in a cell cycle-dependent manner, with binding of USF resulting in 3-fold induction of luciferase activity. In addition, the Elf-1 transcription factor was shown to bind to the Ets consensus binding site (−61 to −54) and to induce activity 3-fold. To verify these observations, we carried out gel shift assays with wild type and mutant probes from the −34 to −15 region. Four oligonucleotide probes, as shown in Table I, were synthesized and used for gel shift assays with MCF-7 total cell extracts. A specific DNA-protein complex was detected with wild type probe (Fig. 2*B*). The complex binds to repeat 2 and is ablated by mutant forms of this 8-bp sequence. The remaining complexes bind to all probes and most likely represent nonspecific binding. These findings were further confirmed by competition experiments. A 90-fold excess of unlabeled wild type oligonucleotide probe effectively blocked binding of labeled probe to the protein complex but had little effect on the nonspecific complexes (Fig. 2*C*). To verify that this protein complex contained a member of the USF transcription factor family, as previously suggested, supershift assays were performed using specific antibody against USF1. USF1 antibody efficiently supershifted the complexes formed with the wild type and M-1 DNA probes. In comparison, antibodies against c-Myc and ATF failed to

TABLE I
Oligonucleotides used for gel shift assay

Designation	Sequence[a]
Wild type sense	GTCGCGTCACGGCGTCACGTGGCCAG
Wild type antisense	CTGGCCACGTGACGCCGTGACGCGAC
M-1 sense	GT**GACAGTAGA**GCGTCACGTGGCCAG
M-1 antisense	CTGGCCACGTGACGC**TCTACTGTC**AC
M-2 sense	GTCGCGTCACGG**TACACTATT**GCCAG
M-2 antisense	CTGGC**AATAGTGTA**CCGTGACGCGAC
M-1+2 antisense	CTGGC**AATAGTGTA**C**TCTACTGTC**AC
M-1+2 sense	GT**GACAGTAGA**G**TACACTATT**GCCAG

[a] All sequences are written in the 5′ to 3′ direction, and sequences of repeat 1 and repeat 2 are underlined. Mutated sequences are indicated in bold type.

supershift the complex (Fig. 2*D*). The combined data strongly suggest that USF binds to the BRCA2 promoter.

To address the role of USF in regulation of BRCA2 basal transcription, a series of expression assays were performed. Cotransfection of USF1 or USF2 expression constructs with the Del-15 luciferase reporter construct had no significant effect on luciferase activity in MCF-7 (data not shown). As a control, CREB, c-Myc, and c-Myc plus Max expression constructs were also cotransfected with the reporter constructs into the various cell lines. Ectopic expression of these transcription factors failed to induce luciferase activity (data not shown). Recent studies of USF-dependent promoters containing USF consensus binding sites in a variety of cell lines have determined that USF cooperates with transactivating factors to induce expression. In fact, ectopic expression of USF1 or USF2 in most epithelial tumor cell lines, such as MCF-7, results in minimal induction of USF-dependent promoters (28). However, in normal mammary epithelial cell lines such as human mammary epithelial cells (Clonetics) and MCF10A, and in the Saos-2 osteosarcoma cell line, ectopic expression of USF1 or USF2 induced a substantial increase in reporter gene expression and activity (28, 29), suggesting a requirement for a cell line-specific transactivating factor. To evaluate whether USF must interact with a transactivating partner to induce the BRCA2 promoter, we ectopically expressed a USF-VP16 fusion construct in MCF-7 cells. As shown in Fig. 2*E*, the USF-VP16 fusion protein induced a 4-fold increase in luciferase activity from the full-length BRCA2 promoter and from the minimal promoter (Del-15). In addition, mutations in the repeat 2 USF binding site ablated the increased luciferase activity. This suggests that USF interacts with other transactivating proteins to regulate basal transcription from the BRCA2 promoter.

Induction of the BRCA2 Promoter by NFκB—Following validation of the role of USF in regulation of BRCA2 basal transcription, we began to systematically map other transcription factor binding sites within the BRCA2 promoter that contribute to regulation of the promoter. Initially, we focused on the −144 to −59 region that was shown to induce basal transcription 3-fold. Sequence analysis of this region identified several putative transcription factor binding sites including an NFκB consensus binding site located at positions −116 to −107 in the 8-kb BRCA2 promoter. To examine the role of NFκB in regulation of the BRCA2 promoter, the effect of overexpression of NFκB on luciferase activity was studied. Cotransfection of expression constructs of the p65 and p50 subunits of NFκB with the pGL3Prom reporter construct containing the wild type BRCA2 promoter resulted in significant induction of luciferase activity. Expression of p65 alone and in combination with p50 increased activity 9- and 16-fold, respectively (Fig. 3*A*). However, expression of p50 alone resulted in a small reduction in activity in comparison to a vector control.

To determine whether this NFκB site was required for regulation of BRCA2 basal transcription, the consensus GGAATTTCCT site was substituted by TAACTTTCCT in the Del-14 BRCA2 promoter reporter construct. The Del-14 construct and the Del-14 mutant construct were transfected into MCF-7 cells, and the luciferase activity was measured as before. As shown in Fig. 3*B*, expression of p65 or p65 with p50 induced a 3–6-fold increase in luciferase activity from the wild type Del-14 promoter in MCF-7 cells but had little activating effect on the mutant promoter. These data suggest that the NFκB p65 subunit can induce BRCA2 promoter activity by forming a heterodimer with endogeneous or ectopically expressed p50.

NFκB Binds to BRCA2 Promoter—To determine whether NFκB subunit proteins bind to the NFκB site in the BRCA2 promoter, we performed gel shift assays of MCF-7 whole cell protein extracts with wild type (WT-κB) and mutant (MT-κB) oligonucleotide probes containing the NFκB site from the BRCA2 promoter (Table II). Whole cell extracts were prepared from MCF-7 cells 48 h after transfection with pcDNA 3.1 vector and with NFκB p65 plus p50 expression constructs. Gel shift analysis demonstrated that a protein complex specifically binds to the wild type NFκB probe but not to the mutant probe following overexpression of p50 and p65 (Fig. 3*C*). No significant complex formed in the absence of overexpression of these genes. Addition of 100-fold excess of cold competitor DNA probe completely eliminated protein binding to labeled DNA probe (data not shown), suggesting that the protein complex binds specifically to the NFκB site in the BRCA2 promoter. The complex was also supershifted by anti-p50 antibody, indicating that the NFκB p50 subunit formed part of the complex (Fig. 3*C*). Although an anti-p65 antibody did not supershift the complex, a significant decrease in the amount of labeled complex was observed (Fig. 3*C*). Thus, the anti-p65 antibody may be binding to p65 in the complex, resulting in reduced access of the DNA probe to the p50 DNA-binding subunit of NFκB. These data suggest that a p50/p65 NFκB heterodimer directly interacts with the NFκB-like site in the BRCA2 promoter, resulting in direct induction of the promoter.

In Vivo Induction of BRCA2 by Overexpression of NFκB—To demonstrate an *in vivo* effect of NFκB on BRCA2 promoter function, we studied the effect of overexpression of p50 and p65 NFκB subunits on endogeneous BRCA2 expression. As before, p50 and p65 constructs were transfected into MCF-7 cells, and Northern blots of RNA from the cells were hybridized with a full-length BRCA2 cDNA probe. Substantial increases in BRCA2 mRNA expression were observed following ectopic expression of p65 and p50 plus p65 (Fig. 4*A*). In addition, Western blots of whole cell extracts were hybridized with anti-p50 and anti-p65 antibodies (Santa Cruz Biotech) to verify expression of the NFκB subunits and with 9D3 anti-BRCA2 antibody (GeneTex) to determine protein levels of BRCA2 in response to expression of NFκB subunits. Transfections of MCF7 cells with p65 and p50 plus p65 constructs resulted in substantially increased levels of these proteins (Fig. 4*B*). BRCA2 protein levels were also significantly increased in response to p65 and p50 plus p65 expression, whereas BRCA2 levels remained low in vector control transfected cells (Fig. 4*B*). This result verifies that NFκB expression results in induction of BRCA2 expression.

Dominant Negative and Wild Type IκBα Inhibit NFκB-dependent Induction of BRCA2—To further demonstrate the role of NFκB in regulation of the BRCA2 promoter, the effect of signaling from the NFκB signaling pathway on BRCA2 promoter induction was assessed. In this study, transfection with a wild type IκBα or a dominant negative mutant IκBα expression construct was used to block signaling through the NFκB

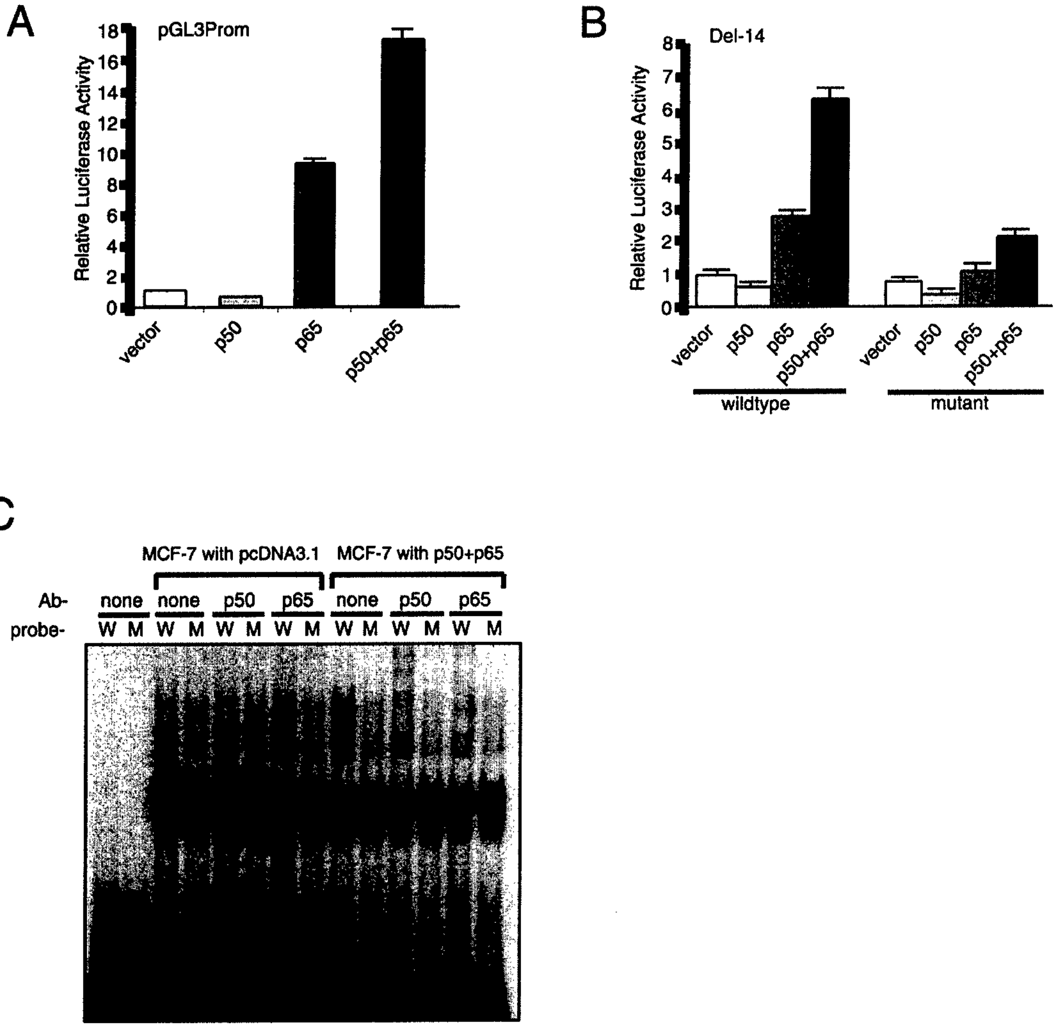

FIG. 3. **Nuclear factor κB induces the BRCA2 promoter.** *A*, ectopic expression of the p65 and p50 NFκB subunits activates the pGL3Prom wild type BRCA2 promoter in MCF-7 cells. The pGL3Prom wild type BRCA2 promoter reporter gene construct was transfected into MCF-7 cells with a pRL-TK *Renilla* luciferase construct and either pcDNA 3.1 (vector), p50 expression construct (p50), p65 expression construct (p65), or p50 and p65 expression constructs (p50+p65). Luciferase activities were normalized by the *Renilla* luciferase activity and are presented relative to the pcDNA3.1 control. *B*, ectopic expression of the p65 and p50 NFκB subunits activates the minimal BRCA2 promoter in MCF-7 cells. The Del-14 wild type and Del-14 mutant (substitution in the NFκB consensus binding site) reporter constructs were transfected with p50 and p65 expression constructs or a vector control. Luciferase activity was normalized as before and is shown relative to activity from the Del-14 wild type. *C*, NFκB p50/p65 heterodimers bind to the NFκB consensus binding site in the BRCA2 promoter in MCF-7 cells. Gel shift assays for the p50 and p65 NFκB subunits with wild type (*W*) and mutant (*M*) oligonucleotide probes for the NFκB consensus binding site in the BRCA2 promoter are shown. Gel shifts were performed using extracts from pcDNA3.1 or p65 and p50 transfected MCF-7 cells. Supershift assays were performed with anti-p50 and anti-p65 antibodies.

TABLE II
Oligonucleotides used for DNA binding assay of NF-κB

Designation	Sequence[a]
WT-κB sense	TTGGGATGCCTGACAAGGAATTTCCTTTCGCCACACT
WT-κB antisense	AGTGTGGCGAAAGGAAATTCCTTGTCAGGCATCCCAA
MT-κB sense	TTGGGATGCCTGACAA**TAACTTTCCT**TTCGCCACACT
MT-κB antisense	AGTGTGGCGAA**AGGAAAGTTA**TTGTCAGGCATCCCAA

[a] All sequences are written in the 5′ to 3′ direction, and the sequence of the nuclear factor κB binding site is underlined. Mutated nuclear factor κB-like site is indicated in bold type.

pathway. The dominant negative IκBα mutant (IκBα 32A/36A) (30) is mutated at two phosphorylation sites and cannot be degraded following IKK-dependent phosphorylation, resulting in retention of NFκB in the cytoplasm. MCF-7 cells were co-transfected with expression constructs for p65, dn-IκBα, IκBα, p65 plus dn-IκBα, p65 plus IκBα, and vector controls along with the pGL3Prom reporter construct. Expression of dn-IκBα or IκBα in combination with p65 resulted in a significant reduction in luciferase activity when compared with the effect of p65 alone, as shown in Fig. 5*A*. In addition, quantitative reverse transcription-PCR analysis of RNA from these transfected cells demonstrated that ectopic expression of dn-IκBα or IκBα significantly reduced the level of expression of BRCA2 (Fig. 5*B*). These data suggest that inhibition of nuclear translocation of NFκB by dn-IκBα or IκBα substantially inhibits BRCA2 promoter activity.

DISCUSSION

Evidence for involvement of BRCA2 in regulation of cellular response to DNA damage (9, 12), in cell proliferation (8), in cell cycle regulation (18), and in transcriptional regulation (31–33) has been accumulating. The variety of functions of BRCA2 suggests that regulation of expression levels of this gene may play an important role in regulation of a number of important cellular processes and that alterations in BRCA2 expression may contribute to tumorigenesis. Interestingly, although no somatic mutations have been identified in BRCA2, apparent overexpression of BRCA2 has been detected in a significant proportion of sporadic breast cancers.

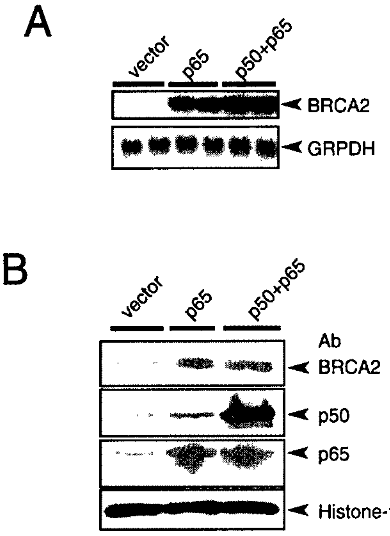

FIG. 4. **Expression of NFκB subunits results in increased BRCA2 expression.** *A*, overexpressed NFκB subunits induce BRCA2 mRNA expression. Total RNA isolated from MCF-7 cells 48 h after transfection with vector, p65, or p65 plus p50 was Northern blotted with [α-^{32}P]ATP-labeled human BRCA2 cDNA probe. The level of BRCA2 mRNA was normalized by GAPDH. *B*, overexpression of NFκB subunits increases BRCA2 protein levels. Cell lysates isolated from MCF-7 cells 48 h after transfection with vector, p65, or p65 plus p50 were prepared as described above and used for Western blotting with primary antibodies against BRCA2, p50, p65, or a histone-1 control. *Ab*, antibody.

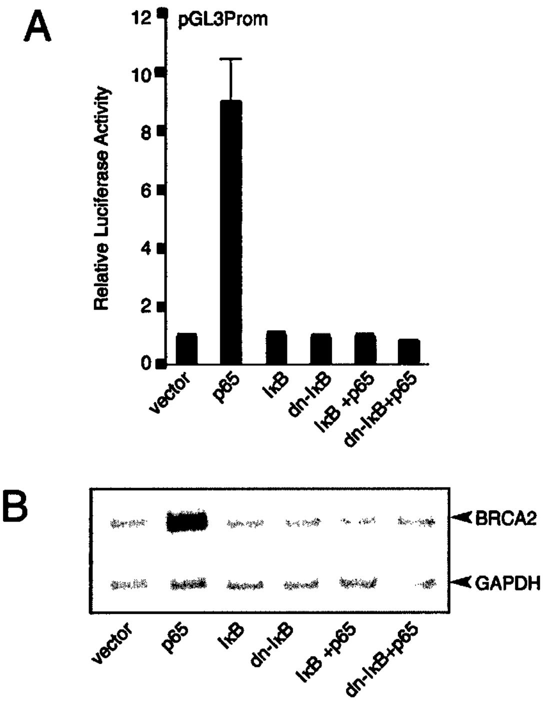

FIG. 5. **Inhibition of the NFκB signaling pathway prevents induction of the BRCA2 promoter.** *A*, wild type and dominant negative mutant IκBα block NFκB-dependent induction of the BRCA2 promoter. MCF-7 cells were cotransfected with IκBα, dn-IκBα, p65, dn-IκBα plus p65, IκBα plus p65, and vector controls along with the pGL3Prom BRCA2 promoter luciferase reporter. After 48 h luciferase activity in whole cell lysates was measured and normalized against *Renilla* luciferase activity. Luciferase activity relative to the vector control is shown for each transfection. *B*, wild type and dominant negative mutant IκBα inhibit NFκB-dependent BRCA2 expression. RNA prepared from the transfections in *A* was used for quantitative reverse transcription-PCR of BRCA2. PCR products for BRCA2 and the GAPDH normalization control for each transfection are shown.

In this study, we have shown that the NFκB transcription factor binds to the BRCA2 promoter and induces expression of the BRCA2 gene. Deletion mapping of the promoter determined that the −144 and −59 region, which contains an NFκB binding site (GGAATTTCCT), is associated with 3-fold activation of the promoter. A combination of gel shift and supershift assays confirmed that NFκB binds to the NFκB *cis*-element. In addition, ectopic expression of NFκB subunits p65 or p65 plus p50 resulted in induction of the BRCA2 promoter and increased levels of BRCA2 mRNA and protein within MCF-7 cells, whereas substitution mutations in the NFκB binding site ablated these effects. These data strongly suggest that NFκB can activate the BRCA2 promoter and induce increased expression of the BRCA2 gene.

The NFκB transcription factor consists mostly of p50/p65 heterodimers, which are complexed to IκBα in the cytoplasm of unstimulated cells. Upon activation of the NFκB signaling pathway, degradation of IκBα exposes nuclear localization signals on the p50/p65 heterodimer leading to nuclear translocation and transcriptional activation of a number of promoters. In this study, we have shown that overexpression of the p50 DNA-binding domain of NFκB does not result in up-regulation of the BRCA2 promoter. Ectopically expressed p50 most likely forms a heterodimer with endogeneous p65, but because p65 levels are low and the NFκB nuclear localization signals are present in p65, relatively little heterodimer translocates to the nucleus and binds to the BRCA2 promoter. Conversely, expression of the p65 subunit with or without ectopic p50 significantly induced luciferase activity, suggesting that the transactivating p65 subunit is necessary for induction of the BRCA2 promoter. Overexpressed p65 most likely binds to endogeneous p50, saturates IκB, and translocates to the nucleus resulting in up-regulation of the BRCA2 promoter. In this case, endogeneous levels of p50 appear to be sufficient to facilitate increased binding of the p50/p65 heterodimer to the promoter. Although only the p50 and p65 NFκB subunits were analyzed in this study, it is likely that the other subunits such as c-Rel, p52, and RelB are also capable of contributing to induction of the BRCA2 promoter.

To verify the role of NFκB in BRCA2 transcriptional regulation we also evaluated the effect of the NFκB signaling pathway on BRCA2 expression. IκB is a component of the NFκB signaling pathway that binds to NFκB and prevents nuclear translocation of NFκB. Thus, overexpression of IκBα or a dominant negative form of IκBα that is resistant to IKK dependent degradation is expected to inhibit NFκB nuclear translocation and NFκB dependent promoter induction. In this study, ectopic expression of both dn-IκBα and IκBα abrogated NFκB-dependent BRCA2 promoter induction and down-regulated BRCA2 mRNA levels, suggesting that expression of the BRCA2 tumor suppressor can be regulated by modulation of the NFκB signaling pathway.

NFκB is known to regulate expression of a large number of genes that play critical roles in regulation of apoptosis, tumorigenesis, and inflammation. In breast cancers, alterations in DNA binding activity, gene expression, and/or nuclear translocation of NFκB proteins have been observed. More specifically, increased NFκB DNA binding activity has been correlated with expression of the *c-erbB*-2 gene (34), and high levels of NFκB/Rel binding have been observed in carcinogen-induced primary rat mammary tumors (35). Because NFκB appears to regulate BRCA2 expression, it seems likely that alterations in NFκB expression and DNA binding (34, 35) contribute to the observed overexpression of BRCA2 in breast tumors (26). Thus, alteration of expression of the BRCA2 tumor suppressor gene may be one mechanism by which aberrantly regulated NFκB contributes to tumorigenesis.

Interestingly, a 3-fold difference in luciferase activity between the pGL3Prom construct and the Del-14 construct was detected in the presence of ectopically expressed p65. This result suggests that other elements within the BRCA2 promoter are directly or indirectly responsive to NFκB. One other NFκB consensus binding site (GAGAAACCCC) was identified in the promoter at positions −808 to −799. However, using deletion constructs and by overexpressing p65, we have shown that this NFκB site does not play a role in regulation of the BRCA2 promoter (data not shown). Thus, other *cis*-elements that are indirectly affected by NFκB may contribute to regulation of the BRCA2 promoter.

In addition to NFκB responsive elements, we have also identified another activation domain that results in 3-fold reduction in activity when removed and a single repression domain that results in 2.5-fold activation when removed (Fig. 1*B*). The transcription factor binding sites from these regions and the associated transcription factors that contribute to regulation of the BRCA2 promoter are not yet known. Recently a repression domain associated with 10-fold down-regulation of the BRCA2 promoter was reported (36). This domain is associated with two Alu repeats and is located in the Del-7 and Del-8 clones and is deleted from the Del-9 clone shown in Fig. 1. However, in the current study the repression domain in the BRCA2 promoter maps to a different location (Del-9; Fig. 1). Further studies are needed to explain the differing results from the two studies.

The role of the USF transcription factor in regulation of basal transcription from the BRCA2 promoter was also verified in the course of this study. A critical 20-bp regulatory sequence (−34 to −15), which is predominantly controlled by binding of USF and is responsible for the majority of BRCA2 transcription, was identified. The critical 20-bp region contains a tandem repeat sequence (GCGTCACG) (Fig. 2*A*) and consensus DNA-binding motifs for transcription factors such as c-Myc, ATF, and USF. Gel shift, supershift, and cotransfection studies demonstrated that only USF binds to the second repeat and regulates the BRCA2 promoter. Recently, Davis and colleagues (15) reported that USF binds to the BRCA2 promoter as a heterodimeric complex of USF-1 and USF-2 and regulates basal transcription in a cell cycle-dependent manner. In this earlier study, overexpression of USF in MCF-7 cells induced only a 2.5-fold increase in promoter activity. However, the addition of IE62, a varicella zoster viral protein that binds to USF proteins (37), resulted in 12-fold induction of promoter activity. Similarly, we have demonstrated that expression of a USF-VP16 fusion protein enhanced induction of the promoter. These results suggest that a co-activating factor is needed for USF activation of the BRCA2 promoter.

The USF family of basic helix-loop-helix leucine zipper transcription factors were originally named MLTF because of their involvement in transcription from the adenovirus major later promoter (38). It is noteworthy that many USF target genes such as p53 (39), transforming growth factor β2 (40), and cyclin B1 (41) are involved in regulation of proliferation and the cell cycle. Moreover, USF overexpression significantly inhibits c-Myc-dependent cell transformation (42) and proliferation of certain transformed cells (29). Thus, the activation of the BRCA2 tumor suppressor gene promoter by USF is consistent with the anti-proliferative effect of this transcription factor. The observation that USF transcriptional activity is lost in breast cancer cell lines but not in normal breast epithelial cells (28) further supports a role for USF as a key regulator of breast cancer development. The combination of these studies and our data suggests that regulation of BRCA2 promoter activity by USF may serve an essential role in the prevention of breast cancer development.

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PROPRIETARY INFORMATION

Repression of BRCA2 transcription by adriamycin and p53 is USF dependent

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ABSTRACT

Adriamycin and other DNA damaging agents have been shown to reduce BRCA2 mRNA levels in breast cancer cell lines. In this study, we show that the reduction in BRCA2 mRNA levels is a result of repression of the BRCA2 promoter, and that the effect is dependent on the presence of wildtype p53. Elimination of p53 in p53 mutant or null cell lines, by expression of human papilloma virus E6, or by expression of a dominant negative p53 construct inhibited adriamycin associated repression of the BRCA2 promoter. In contrast, ectopic expression of wildtype p53 resulted in repression of BRCA2 promoter activity, and a reduction in BRCA2 mRNA and protein levels. Both adriamycin and p53 mediated repression of the promoter by reducing USF dependent activation of the promoter and by inhibiting binding of a USF protein complex to the USF consensus binding site in the promoter. In addition, ADR and p53 repressed promoter activity in a cell cycle independent manner. These results suggest a regulatory loop in which BRCA2 inhibits p53 dependent transcription, and p53 represses BRCA2 expression in response to DNA damage.

INTRODUCTION

The BRCA2 gene was identified in 1996 as a breast and ovarian cancer susceptibility gene (1,2). The BRCA2 gene encodes a 3418 amino acid cell cycle regulated nuclear phosphoprotein (2-4) that has been implicated in gene transcription and the response to DNA damage. Evidence for a role in transcription comes primarily from identification of P/CAF as a BRCA2 binding protein, and the associated delineation of a transactivation domain at the N-terminus of BRCA2 (5). The evidence for a role in DNA repair is far more substantial. BRCA2 binds directly with RAD51 through the exon 11 encoded BRC repeats (6,7), and to mouse RAD51 through a C-terminal binding site (8). This association with a protein involved in meiotic and mitotic recombination and DNA double-strand break repair suggests a similar role for BRCA2. Further support for a role in DNA repair comes from the observation that cells expressing a wildtype BRCA2 BRC4 domain show hypersensitivity to γ-irradiation, an inability to form RAD51 radiation-induced foci, and a failure of radiation-induced G_2/M, but not G_1/S, checkpoint control (9). Moreover, cells expressing mutant BRCA2 are more sensitive to methyl methanesulfonate-induced DNA damage than cells expressing wildtype BRCA2 (10). Animal models have also been used to demonstrate an association between BRCA2 and the DNA damage response. BRCA2 null mouse embryos that do not survive past day 8 of embryogenesis are highly sensitive to γ-irradiation (8). Similarly, mouse embryo fibroblast (MEF) cell lines derived from viable $BRCA2^{-/-}$ animals are highly sensitive to DNA damage induced by a number of agents (11,12). Most recently, BRCA2 has been directly implicated in homologous recombination and gene conversion using CAPAN-1 BRCA2 mutant cell lines and homozygous mutant BRCA2 ES cells (13,14).

BRCA2 also appears to be functionally associated with the p53 protein. The survival of homozygous mutant BRCA2 embryos that are non-viable after day 7 of development can be extended to 10 days by the presence of a p53 inactivating mutation (15). This suggests that cells with nonfunctional BRCA2 can only survive if they also have defective p53 dependent checkpoint control. In addition, a recent study has shown that BRCA2 physically interacts with p53 in a RAD51/p53 complex and partially represses p53 dependent transactivation of target promoters such as $p21^{Waf1/CIP1}$(16).

Furthermore, a large proportion of BRCA2 associated breast tumors contain mutations in the p53 gene (17) suggesting that p53 inactivation may be a critical step during tumorigenesis in BRCA2 carriers. These functional associations between BRCA2 and p53 suggest that these proteins may work together to regulate genomic stability

The effect of DNA damage on BRCA2 protein has also been studied. Treatment of MCF7 breast cancer cell lines with DNA damaging agents such UV light, adriamycin (ADR, a topoisomerase II inhibitor), and camptothecin (a topoisomerase I inhibitor) results in significant downregulation of BRCA2 mRNA levels (18). ADR and UV light also down regulate BRCA2 mRNA levels in ovarian cancer cell lines in a dose and time dependent manner (19). In this study we describe the mechanism by which ADR downregulates expression from the BRCA2 promoter. We show that the effect is dependent on wildtype p53, and that mutant forms of p53 inhibit the repressive effect of ADR on the promoter. Furthermore, we demonstrate that ADR and p53 repress BRCA2 promoter activity by inhibiting binding of the USF transcription factor to the minimal promoter. Thus, while BRCA2 can repress the transactivating potential of p53 (16), p53 can also repress BRCA2 expression.

MATERIALS AND METHODS

Plasmids. The preparation of luciferase reporter gene constructs in the pGL3 vector (Clontech) has been previously described (20). A pGL13 luciferase reporter construct containing thirteen-consensus p53 binding sequences was generously provided by Wafik El-Deiry. A pcDNA3.1 plasmid containing the wild type p53 cDNA (wtp53) was provided by Wilma Lingle. A dominant negative p53 mutant construct (R273Lp53, dnp53) was generated by site-directed mutagenesis of the wtp53 construct using the Quikchange kit (Qiagen) according to the manufacturer's instructions. PCR primers for the site-directed mutagenesis were 5'-GGAACAGCTTTGAGGTGCTTGTTTGTGCCTGTCCTGG-3' (forward) and 5'-CCAGGACAGGCACAAACAAGCACCTCAAAGCTGTTCC-3' (reverse). Mutagenesis was performed using *pfu* DNA polymerase and 40ng of wtp53 as template under the following conditions: 1 cycle for 30 sec at 95 C; 12 cycles at 95 °C for 30 sec, 55 °C for 1 min, 68 °C for 14 min. The PCR product was digested with *Dpn I* for 2 hours and then transformed into *E. coli.* Plasmid DNA was isolated from colonies and the presence of the mutations was confirmed by DNA sequencing.

Cell culture. All cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD). Human breast adenocarcinoma MCF7 cells, human colon carcinoma, SW480 cells and human osteosarcoma U2OS cells were cultured at 37 °C in 5% CO_2 in Dulbecco's Eagle Medium (DMEM) supplemented with 10% bovine calf serum (BCS) (HyClone), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Human osteosarcoma p53-null Saos-2 cells (21) were propagated in McCoy's 5A medium supplemented with 15% BCS, 100 units/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C in 5% CO_2. MCF7/pCMV and MCF7/E6 (22) cells were maintained in RPMI-1640 with 10% BCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cell culture reagents were purchased from Life Technologies.

Transient transfection assays. Transient transfections were performed in 6-well plates using Fugene-6 reagent (Boehringer-Mannheim) with 1.0-2.0 μg of BRCA2 promoter luciferase reporter construct and 0.1 μg of pRL-TK-Renilla luciferase vector (Promega). For ADR (Sigma) treatment experiments, cells were transfected, grown for 24 hr, and exposed to the 0, 2.5, 5.0, 10 or 15 μM amount of ADR for 1 hr in standard media. The cells were washed with serum-free medium and incubated at 37 °C in fresh culture medium for another 24 hr. In co-transfection experiments, cells were also transfected with 0.025-0.5 μg of wtp53, dnp53, USF-VP16, USF1, or pcDNA3.1 control. After 48 hr, protein lysates were prepared from the cells, and luciferase activities were measured as previously described (20).

Electrophoretic mobility shift assays (EMSAs). Double strand oligonucleotides containing the wild type and mutated USF binding site (-26 to -18) in the BRCA2 promoter were labeled with [γ-^{32}P]-ATP and used in EMSA's (20). Double stranded DNA probes were purified from the reaction mixture using a Bio-Gel P100 column (Bio-Rad), incubated with whole cell extract from MCF7 cells, and separated on 5% polyacrylamide gels as previously described (20). Super shift assays using anti-p53 (Santa Cruz), USF1 (Santa Cruz), and USF2 (Santa Cruz) antibodies were also performed as previously described (20).

RNA isolation and northern blotting. Saos-2 and MCF7 cells were transiently transfected with pcDNA 3.1, wtp53, or dnp53 expression constructs and/or exposed to 5 μM ADR for 1 hr. After 24 hr further incubation, poly A^+ RNA was isolated. RNA samples (1.5 μg/lane) were used for Northern blotting as previously described (20).

Western blotting. MCF7 cells that were transiently transfected with plasmids and/or treated with 5 μM ADR for 1 hr were grown for 24 hr. Cell lysates were prepared by standard methods and western blotted with anti-BRCA2 (Oncogene Research) (23), anti-USF1 (Santa Cruz) and anti-p53 (Santa Cruz) antibodies and alkaline phosphatase - conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Signals were developed using the ECL detection system.

Flow cytometry. MCF7 cells treated with ADR were washed with PBS, collected by centrifugation and fixed in 4% paraformaldehyde for 15 min on ice. After fixation, cells were washed with ice-cold 80% ethanol and 1% FBS. The fixed cells were permeabilized and stained with 0.2% Triton X-100, 100 mg/ml RNase A and 10 mg/ml propidium iodide at 37 °C for 30 min and analyzed by flow cytometry. The data were processed with VERITY ModFit, version 5.2, software for DNA distribution analysis.

RESULTS

The BRCA2 promoter is repressed by ADR in MCF7 cells. ADR has recently been shown to down regulate BRCA2 mRNA levels in human breast cancer cells such as MCF7 (18). To address whether the reduction in BRCA2 mRNA levels by ADR is dependent on BRCA2 promoter regulation, the effect of ADR on luciferase activity from a BRCA2 promoter luciferase reporter was measured. MCF7 cells were transiently transfected with a BRCA2 promoter reporter gene construct (pGL3Prom) containing 8kb of DNA surrounding the BRCA2 transcription start site (20), and were exposed to various concentrations of ADR for 1 hr. After removal of the drug and further propagation for 24 hr, cell lysates were harvested for luciferase reporter gene activity assays. As shown in figure1, treatment of cells with 2.5 μM of ADR reduced promoter activity almost 5-fold relative to untreated cells. Increasing concentrations of ADR further reduced BRCA2 promoter activity in a dose dependent manner, resulting in 10-fold down-regulation after treatment with 5-10 μM ADR. These data suggest that ADR reduces BRCA2 levels by repression of the BRCA2 promoter.

Repression of the BRCA2 promoter by ADR is dependent on p53. ADR is a potent DNA-damaging agent that induces p53 accumulation in wild type p53 expressing cell lines. To verify that ADR influences p53 function in MCF7 cells expressing wildtype p53 (wtp53) (24,25), we assessed the effect of ADR treatment on a p53 dependent luciferase reporter construct, containing 13-repeated consensus p53 DNA binding sites located upstream of the luciferase gene, in these cells. As shown in Fig. 2A, ADR induced luciferase activity and enhanced wtp53 dependent activation of the promoter, suggesting that ADR can enhance p53 dependent transactivation in MCF7 cells.

Next, we investigated whether ADR down-regulates the BRCA2 promoter in a p53 dependent manner. MCF7 cells were transfected with wtp53, a dominant negative form of p53 (dnp53) (R273L), or a pcDNA 3.1 vector control, and were treated with 5 μM ADR for 1 hr. In pcDNA3.1 transfected cells, BRCA2 promoter activity was inhibited 5-fold by ADR exposure (Fig. 2B). However, promoter activity was reduced 10-fold in cells transfected with wtp53 in the absence of ADR, suggesting that wtp53 can also repress the BRCA2 promoter. Treatment of wtp53 transfected cells with 5 μM ADR repressed the promoter a further 2-fold for a total of 20-fold down-regulation (Fig. 2B). Interestingly, ADR and wtp53 did not have a synergistic effect on the promoter, suggesting that they influence the BRCA2 promoter through a common signaling pathway. In contrast to wtp53, expression of the dnp53 mutant resulted in marked up-regulation of the BRCA2 promoter in the absence of ADR. However, the addition of 5 μM ADR reduced this effect so that the combination of dnp53 and ADR displayed a slight repressive effect (Fig. 2B). This was possibly due to competition between dnp53 and wtp53 induced by ADR. Expression of the wtp53 and dnp53 proteins in the MCF7 cells was verified by western blot with anti-p53 monoclonal antibody (data not shown).

Having demonstrated that ectopic expression of wtp53 can repress the BRCA2 promoter, the dose-dependency of this effect was evaluated by co-transfection of varying amounts of wtp53 into MCF7 cells with the BRCA2 promoter reporter construct. As shown in Fig. 2C, increasing concentrations of wtp53 resulted in greater levels of repression of the promoter, suggesting a specific effect of wtp53 on the BRCA2 promoter.

To determine if endogeneous p53 is required for regulation of the promoter, we repeated the reporter assays in p53-positive U2OS and p53-null Saos-2 cells. As both cell lines are derived from osteosarcomas, this experiment is expected to address the role of p53 in regulation of the promoter, while minimizing tissue specific differences. In p53-null Saos-2 cells, ADR did not affect BRCA2 promoter activity, while in the p53-positive U2OS cells a dose dependent inhibition of the promoter was observed, suggesting that p53 is required for ADR dependent repression of the BRCA2 promoter (Fig. 2D).

To further establish the importance of p53 in regulation of the BRCA2 promoter, the reporter assays were repeated in MCF7 cells stably expressing either the human papilloma virus type 16 (HPV-16) E6 gene (MCF7/E6) or a CMV vector control (MCF7/CMV) (22). E6 protein stimulates degradation of p53 through a ubiquitin pathway (26,27), resulting in very low levels of p53 expression in these cells, as verified by western blotting (data not shown). Upon treatment of these cells with ADR, only the MCF7/CMV cells displayed a dose-dependent decrease in BRCA2 promoter activity (Fig.

2E). Taken together, these data indicate that repression of BRCA2 promoter activity by ADR is p53 dependent.

The ADR-responsive region is adjacent to the transcription initiation site. To identify the ADR responsive region within the 8 kb BRCA2 promoter, a series of luciferase reporter constructs containing deleted and mutated forms of the full length BRCA2 promoter were utilized (Fig. 3A) (20). MCF7 cells were transfected with the various constructs, and luciferase activities were measured before and after treatment with 5 µM ADR. The luciferase activity associated with each construct in ADR treated cells relative to the activity in untreated cells is shown (Fig. 3B). All promoter constructs were repressed by ADR treatment, but the effect was 3-fold less when the -58 to -19 region was deleted (Del-16) (Fig. 3A,B), suggesting that this region mediates the response to ADR. No other region of the promoter demonstrated any substantial response to ADR.

The -58 to -19 region contains a tandem repeat sequence (GCGTCACG), and a consensus USF transcription factor binding site that is critical for basal transcriptional regulation of the BRCA2 promoter (20). In this study, mutation of the 2nd repeat sequence (Del-15-2) or of both repeat sequences (Del-15-3) resulted in a 2-fold reduction in ADR dependent repression of the BRCA2 promoter (Fig. 2B). This suggests that the USF binding site or another overlapping transcription factor binding site mediates the ADR effect on BRCA2 promoter activity.

Because the ADR effect on BRCA2 promoter activity is mediated by p53, as described above, we postulated that the ADR responsive site in the BRCA2 promoter might also respond to p53. To address this hypothesis, wtp53 was co-transfected into MCF7 cells with each of the BRCA2 reporter constructs. The luciferase activity associated with each construct in p53 transfected cells relative to the activity in untransfected cells is shown in figure 3C. As detected after ADR treatment, the -58 to -19 region and more specifically the 2nd repeat sequence containing the USF binding site appeared to be responsible for 2- to 3-fold repression of the promoter. This suggests that the ADR effect on the BRCA2 promoter is modulated by p53.

Wtp53 inhibits binding of USF to the BRCA2 promoter. To determine if the USF transcription factor is directly involved in p53 and ADR dependent regulation of BRCA2 promoter activity, the ability of USF to bind to the BRCA2 promoter was evaluated. Two 26 bp (-10 to -35) oligonucleotide probes containing either a wild type (W) or mutated (M) USF binding site (20) were used in gel shift assays with lysates from MCF7 cells transfected with vector control, wtp53, or dnp53 expression constructs and either exposed or not exposed to 5 μM ADR. As shown in figure 4A, a single specific protein complex from untreated cells bound to the wildtype oligonucleotide. However, the ability of the complex to bind DNA was dramatically decreased when cells were treated with ADR and/or were transfected with wtp53. In contrast, binding of the complex to the wildtype oligonucleotide was stabilized when dnp53 was expressed, even after exposure to ADR. In addition, the specific DNA/protein complex was supershifted by anti-USF1 and anti-USF2 antibodies (Fig. 4B). Together these data suggest that the repressive effects of ADR and p53 on the BRCA2 promoter are mediated by inhibition of USF binding to the promoter DNA. Interestingly, we found that the DNA/protein complex could not be supershifted by anti-p53 antibody (Fig. 4C). This suggests either that p53 is not a part of the USF protein complex, or that the p53 epitope is masked in the complex.

p53 inhibits USF dependent induction of the BRCA2 promoter. To further establish the relevance of USF to p53 and ADR dependent repression of the BRCA2 promoter, we tested whether p53 could block USF-VP16 induction of the BRCA2 promoter. The USF-VP16 fusion protein was utilized because it binds to the USF site and transactivates the BRCA2 promoter in MCF7 cells (20). As shown in figure 5A, expression of USF-VP16 significantly up-regulated the promoter, but this activation was repressed by 40% following ectopic expression of p53. To further confirm that wtp53 represses USF dependent activation of the promoter, the BRCA2 promoter construct was transfected into a dnp53 colon carcinoma cell line (SW480) along with vector control, wtp53, USF1, and wtp53 plus USF1 expression constructs. Ectopic expression of USF1 activated the BRCA2 promoter 4.5 fold in the absence of p53, while expression of wtp53 completely blocked this effect (Fig. 5B). Thus, p53 inhibits USF dependent promoter activation.

***In vivo* repression of BRCA2 expression by ADR requires wtp53.** In order to confirm an *in vivo* effect of ADR on BRCA2 expression, BRCA2 mRNA levels in MCF7 (wtp53) and Saos-2 (p53 null) cells exposed to ADR were measured by Northern blot. The involvement of wtp53 was further evaluated by measuring BRCA2 expression in cells ectopically expressing a vector control, wtp53, or dnp53. Treatment with 5 μM ADR, or overexpression of wtp53 resulted in a substantial decrease in BRCA2 mRNA levels in MCF7 cells (Fig. 6A). However, BRCA2 levels were not substantially affected by ADR treatment in MCF7 cells transfected with dnp53 (Fig. 6A) or p53-null Saos-2 cells (Fig. 6B). The effect of ADR treatment and p53 expression on BRCA2 protein levels was also established by western blotting. As expected, ADR treatment or wtp53 expression decreased BRCA2 levels in MCF7 cells, while dnp53 expression in the presence or absence of ADR treatment had no substantial effect (Fig. 6C). Changes in p53 expression following ADR treatment or transient transfection with wtp53 or dnp53 constructs was also verified by western blotting with anti-p53 antibodies. Furthermore, western blotting with anti-USF1 antibodies demonstrated that USF1 protein levels were not altered in response to ADR or p53 expression. This suggests that regulation of BRCA2 expression by ADR and p53 does not involve reduction in USF1 levels, but is dependent on altered binding of USF1 to the promoter.

Repression of the BRCA2 promoter by ADR and p53 is independent of the cell cycle. It has been suggested in the literature that BRCA2 expression may be cell cycle regulated, with highest levels of protein and mRNA in the S and G_2 phases, and low levels in the G_1 and M phases (3,4). In addition, previous studies have shown that exposure of MCF7 cells to DNA damaging agents such as actinomycin D and ADR results in p53 dependent arrest of the cell cycle at the G_1/S and G_2/M checkpoints (28,29). To determine whether repression of BRCA2 expression by ADR and p53 is associated with cell cycle arrest, the p53 expression level, BRCA2 promoter activity, and cell cycle profile of MCF7 cells were measured at several time-points following ADR treatment. As shown in figure 7, p53 protein levels were substantially increased 6 hr after treatment with ADR, while BRCA2 promoter activity was only slightly repressed. This suggests that p53 indirectly regulates BRCA2 promoter function. At the same time, MCF7 cells were beginning to accumulate in S-phase as a result of an ADR dependent, and perhaps p53 dependent S-phase arrest (Fig. 7C). At the 18 hr timepoint when the promoter was maximally repressed, p53 levels were high, and a large proportion of cells were arrested in S-phase, suggesting that BRCA2 promoter repression might be cell cycle dependent. However, the finding that maximal repression of the promoter occurs when cells are arrested in S-phase after ADR treatment is in direct conflict with prior observations that BRCA2 expression is at its highest when cells are in S-phase of the cell cycle (3,4). This result indicates that BRCA2 promoter activity is regulated by p53 independently of its affect on the cell cycle.

To further determine whether the arrest of the cell cycle is relevant to regulation of the BRCA2 promoter, the role of the p53 dependent, $p21^{Waf1/Cip1}$ cell cycle inhibitor in repression of the BRCA2 promoter was evaluated. The BRCA2 luciferase reporter construct was transiently transfected into HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells (courtesy of Todd Waldmann) and promoter activity was measured following treatment with 5 μM ADR. ADR treatment repressed the BRCA2 promoter activity equivalently in both cell lines, indicating that the ADR and p53 effect is independent of $p21^{Waf1/Cip1}$ and cell cycle regulation. This suggests that any agent or event that induces p53 in the cell will lead to repression of the BRCA2 promoter, regardless of concomitant cell cycle effects.

DISCUSSION

In this study, we show that adriamycin and several other DNA damaging agents including actinomycin D and mitomycin C (data not shown) significantly repress transcription from the human BRCA2 promoter. The inability of these agents to repress the promoter in p53 mutant or null cells, or in cells ectopically expressing the dominant negative R273Lp53 indicates that this is a p53 dependent process. We have extended this observation to show that DNA damage induced p53 prevents binding of the USF transcription factor to its consensus binding site, resulting in down-regulation of the BRCA2 promoter. To our knowledge, this is the first report showing that p53-mediated transcription repression is functionally associated with USF binding. In addition, we have determined that inhibition of BRCA2 promoter activity in response to ADR and p53 is independent of cell cycle regulation.

We have previously shown that USF binds to the BRCA2 minimal promoter and regulates its basal activity (20), and that disruption of the USF binding site reduces promoter activity 5-10-fold. In the present study we demonstrated that ADR and p53 inhibited BRCA2 promoter activity and we mapped the repression site to the same USF binding site. Gel shift assays were used to show that ADR treatment or wtp53 expression inhibited the binding of the USF associated complex to the promoter. In contrast, a dnp53 protein failed to inhibit formation of the USF/DNA complex resulting in mild activation of the promoter rather than repression due to inhibition of endogeneous p53 effects on the USF complex.

While p53 appears to regulate USF binding, it does not seem to bind directly to USF as evidenced by an inability of anti-p53 antibodies to supershift the USF complex. As we have also shown that p53 does not regulate USF expression levels, it appears that p53 may regulate or interact with other protein/s that in turn modulate the ability of USF to bind to the promoter. USF1 has recently been identified as a phosphoprotein (30,31) whose DNA binding activity is dependent on cyclin-dependent phosphorylation (31), and is inhibited by the p53 inducible cyclin dependent kinase inhibitor, $p21^{Waf1/Cip1}$, which blocks phosphorylation of USF1 (31). This suggests that ADR and p53 regulate BRCA2 expression through $p21^{Waf1/Cip1}$. However, we found that p53 and ADR repressed BRCA2 promoter activity in cells lacking $p21^{Waf1/Cip1}$ suggesting that $p21^{Waf1/Cip1}$ plays no role in regulation of the BRCA2 promoter.

Transcriptional repression by p53 has been reported for several genes, including BRCA1 (29), and is thought to be the consequence of p53 dependent inhibition of other transcriptional activators (32-34) or components of the basal transcription machinery (35-37). One mechanism of p53 associated repression utilizes histone deacetylases (HDAC), mediated by interaction with mSin3a, to negatively regulate target genes such as map4 and stathmin (24), and to repress the CHK1 gene through the $p21^{Waf1/Cip1}$ protein (38). Another mechanism of p53 dependent repression involves binding of p53 to p300/CBP and subsequent interference in co-activation of p300/CBP-dependent factors, such as AP-1 (33), hypoxia-inducible factor 1 (39) and NF-κB (40,41). Interestingly, it has been reported that p300 interacts functionally with USF to potentiate the activation of USF target genes (42). Whether the functional interplay between p53 and USF in the BRCA2 promoter regulation is actually mediated by p300/CBP or HDAC or as yet unidentified factors remains to be determined.

While previous reports suggest that ADR treatment induces a G_2/M cell cycle arrest in MCF7 cells (28,29), in this experiment a p53 dependent S-phase arrest was detected. Perhaps this resulted from the treatment of the cells with a single high dose (5 μM) of ADR rather than continuous treatment of cells over 24 hr with a low dose (29). Thus, maximal repression of the BRCA2 promoter in response to ADR and p53 was detected in the phase of the cell cycle that is associated with highest levels of BRCA2 expression in untreated cells. This suggests that p53 can overcome the normal cell cycle regulation of BRCA2 expression and that repression of BRCA2 by p53 is independent of the cell cycle. However, the simultaneous repression of the BRCA2 promoter and induction of an S-phase arrest suggests that these events are in some way associated. Thus, it remains possible that the reduction in BRCA2 levels contributes to p53 dependent S-phase arrest. Further experiments are needed to address this possibility.

There is substantial evidence that BRCA2 plays a role in DNA repair. Therefore, it is surprising that BRCA2 is down-regulated by p53 after DNA damage. One possible explanation is that p53 may down-regulate BRCA2 after the DNA repair functions of BRCA2 are completed. Thus, this may be one mechanism by which p53 regulates the extent, type, and timing of DNA damage repair. Another possibility is that BRCA2 is only required for the earliest response to DNA damage and is actively down-regulated after completing this function. This is supported by the rapid p53 dependent repression of the BRCA2 promoter following DNA damage. Interestingly, BRCA2 forms a complex with p53 and RAD51, and inhibits transactivation of p53 target promoters (16). Thus, the tightly controlled interaction between BRCA2 and p53 may play a critical role in determining whether a cell activates a DNA repair or an apoptotic pathway in response to DNA damage. Further investigations will be needed to improve our understanding of the association between BRCA2 and p53.

In conclusion, we have demonstrated that the BRCA2 promoter is down regulated by ADR, and that the repression effect is p53 dependent and is mediated by altered binding of USF to the proximal promoter. In addition, we have shown that the effect is independent of the cell cycle and can be induced by several other DNA damaging agents. Thus, BRCA2 and p53 share a complex regulatory pathway that appears to be directly associated with the DNA damage response. Further studies are needed to identify the specific mechanism by which p53 inhibits USF binding to the BRCA2 promoter, and to better understand why BRCA2 is down regulated following DNA damage.

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FOOTNOTES

[1] The abbreviations used are: ADR, adriamycin; USF, upstream stimulatory factor; NFκB, nuclear factor-κB; wtp53, wild type p53; dnp53, dominant negative p53; BCS, bovine calf serum; PCR, polymerase chain reaction; FACS, fluorescence activated cell sorting.

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FIGURE LEGENDS

Figure 1. Dose dependent repression of BRCA2 promoter activity by ADR. Luciferase activity (y axis) in MCF7 cells transfected with a BRCA2 promoter reporter construct (pGL3Prom) was measured 24 hr after treatment with varying amounts of ADR for 1 hr (x axis). All luciferase activities were normalized by renilla luciferase activity.

Figure 2. p53 represses the BRCA2 promoter. (A) Wtp53 induces a p53-dependent promoter in MCF7 cells in response to ADR. Luciferase activity from a PG13, p53 dependent artificial promoter, was measured in MCF7 cells transfected with either wtp53 or vector control and treated with 5 μM ADR for 1 hour. Luciferase activity was normalized by renilla luciferase activity. (B) Wtp53 represses BRCA2 promoter activity. Luciferase activity from a BRCA2 promoter reporter construct (pGL3Prom) was measured 24 hr after treatment with 5 μM ADR or DMSO in MCF7 cells transfected with wtp53, dnp53 (R273L), or control vector. (C) Wild type p53 induces a dose-dependent repression on the BRCA2 promoter (pGL3Prom). BRCA2 promoter activity was measured in MCF7 cells transfected with various amounts of a wtp53 expression construct. (D) The inhibitory effect of ADR treatment on the BRCA2 promoter (pGL3Prom) is abolished in p53-null cells. BRCA2 promoter activity was measured in Saos-2 (p53 null) and U2OS (wtp53) cells 24 hr after treatment of the cells with various amounts of ADR. (E) HPV16-E6 degrades p53 and blocks ADR-dependent repression of the BRCA2 promoter. BRCA2 promoter activity was measured in MCF7 cells stably expressing HPV16-E6 or the pCMV control after treatment with various concentrations of ADR.

Figure 3. The adriamycin and p53 -responsive region in the BRCA2 promoter contains a USF binding site. (A) Graphical representation of BRCA2 promoter reporter constructs. The Del-15, Del-15-1, Del-15-2 and Del-15-3 constructs contain wildtype and mutated tandem repeat sequences in the BRCA2 minimal promoter. Substitution mutations are shown with an X. (B) Repression of the BRCA2 promoter by ADR involves the USF binding site. Luciferase activity associated with the various promoter constructs in 5 μM ADR treated MCF7 cells relative to untreated cells is shown. (C)

Repression of the BRCA2 promoter by p53 involves the USF binding site. Luciferase activity associated with the various promoter constructs in wtp53 transfected MCF7 cells relative to control vector transfected cells is shown.

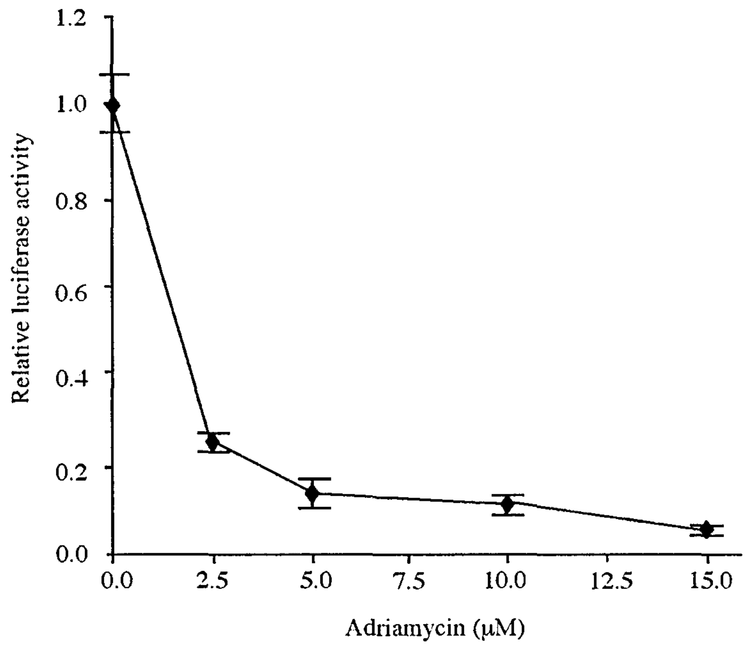
Figure 4. ADR and p53 reduce the DNA binding of USF in the BRCA2 promoter. (A) USF binding to the USF site is inhibited by ADR and wtp53. Electrophoretic mobility shift assays were performed using oligonucleotide probes containing a wild type (W), or mutated (M) USF binding site, and whole cell protein extract from MCF-7 cells transfected with vector control (vector), wtp53 or dnp53 and treated with ADR or DMSO. (B) Wtp53 inhibits binding of USF to the promoter. Anti-USF1 and USF2 antibodies were used to supershift the DNA/protein complex formed at the USF binding site, in the presence and absence of ectopically expressed wtp53. (C) Wtp53 is not associated with the USF/promoter complex. Anti-p53 antibodies failed to supershift the DNA/protein complex formed at the USF binding site.

Figure 5. p53 represses USF dependent induction of the BRCA2 promoter (A) Induction of the BRCA2 promoter by USF-VP16 in MCF7 cells is inhibited by wtp53. Luciferase activity from the BRCA2 promoter reporter was measured in MCF7 cells transfected with combinations of USF-VP16, wtp53, dnp53, and control vector. (B) Induction of the BRCA2 promoter by USF1 in SW480 (p53 mutant) cells is inhibited by wtp53. Luciferase activity from the BRCA2 promoter reporter was measured in SW480 cells transfected with combinations of USF1, wtp53, and control vector.

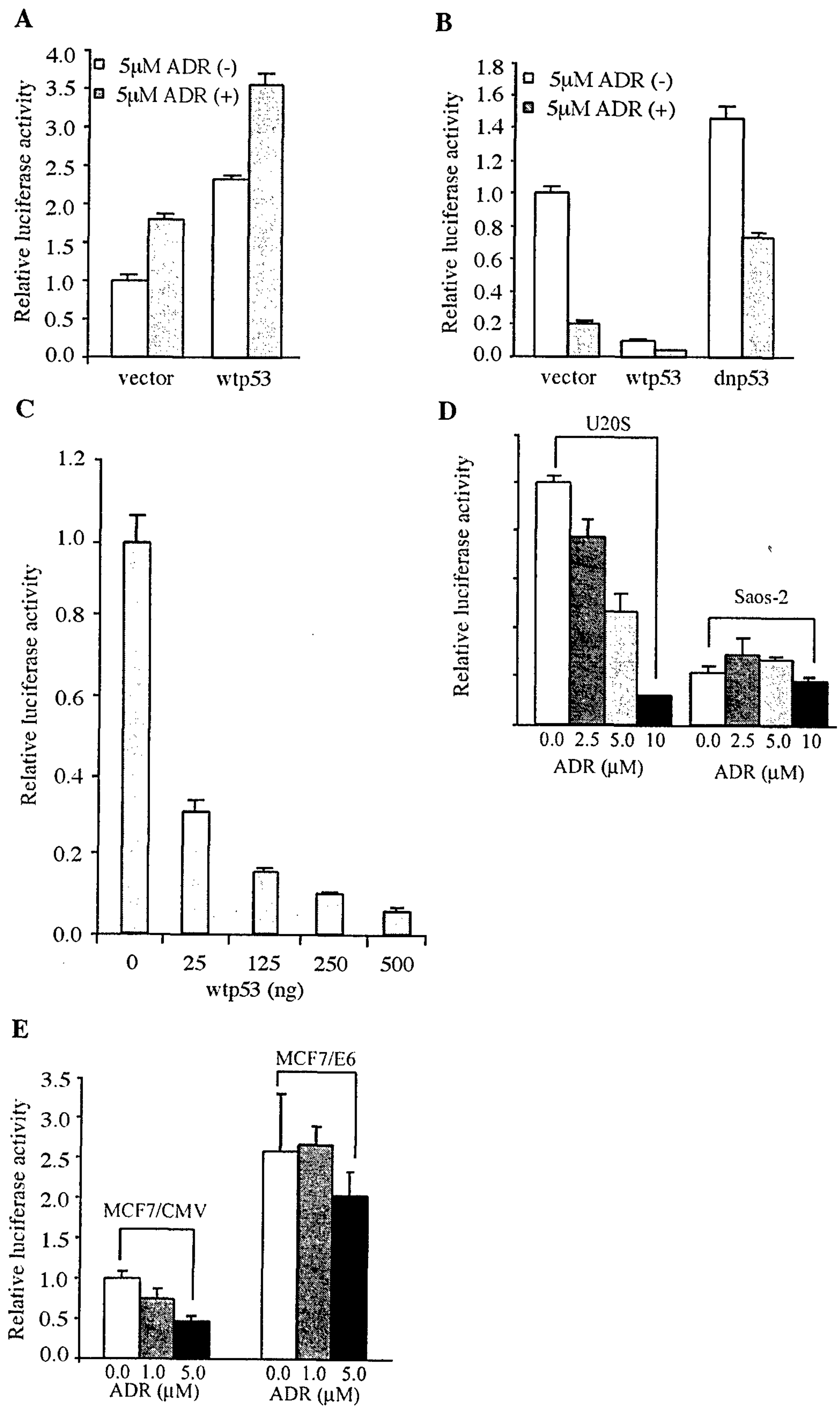
Figure 6. ADR and p53 repress BRCA2 expression in vivo. (A) wtp53 down regulates BRCA2 mRNA expression. BRCA2 expression levels in MCF7 cells transfected with wtp53, dnp53, or vector control and treated with ADR or DMSO were measured by Northern blot of poly A^+ RNA. A GAPDH control probe was used to demonstrate equal loading. (B) ADR does not influence BRCA2 expression in p53-null cells (Saos-2) compared to p53 wildtype MCF-7 cells. BRCA2 expression levels in Saos-2 and MCF7 cells exposed to 5 μM ADR were measured by Northern blot of poly A^+

RNA. (C) ADR and wtp53 reduce BRCA2 protein levels in MCF7 cells. BRCA2 protein levels in MCF7 cells transfected with wtp53, dnp53, or vector control and treated with ADR or DMSO were measured by western blot using an anti-BRCA2 antibody (23). Blots were probed with anti-p53 (Santa Cruz) antibody to demonstrate expression of p53 after transfection or ADR treatment. Blots were also probed with anti-USF1 antibody (Santa Cruz) to demonstrate that USF1 was not induced by wtp53 or ADR, and histone-1 antibody was used to show equal loading.

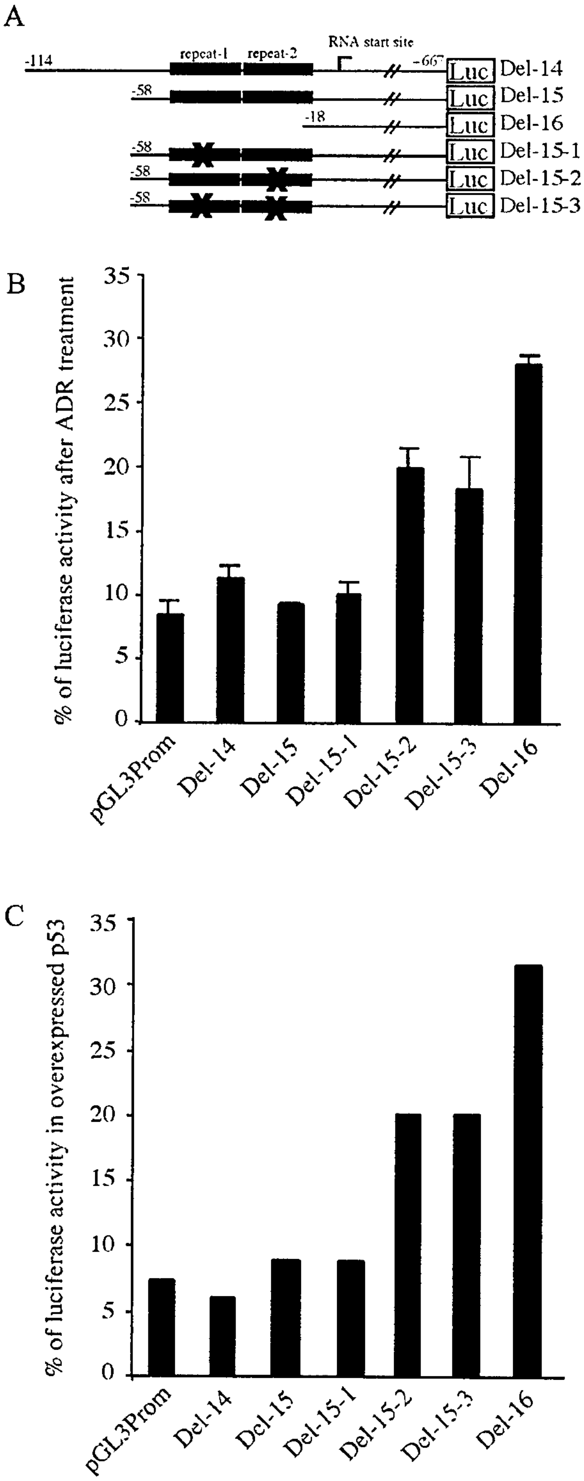
Figure 7. Repression of the BRCA2 promoter coincides with an S-phase cell cycle arrest. (A) Time dependent repression of the BRCA2 promoter. Luciferase activity from the BRCA2 promoter reporter in MCF7 cells is shown at different time points following ADR treatment. Activity at each time point is relative to activity at time 0 hr. (B) Time dependent induction of p53 in response to ADR treatment. Western blots of p53 with anti-p53 antibody at different time points following ADR treatment. (C) Cell cycle profile of MCF7 cells at different time points following treatment with ADR. The percentage (%) of cells in each phase of the cell cycle at each time point was determined by FACS analysis of PI stained MCF7 cells.



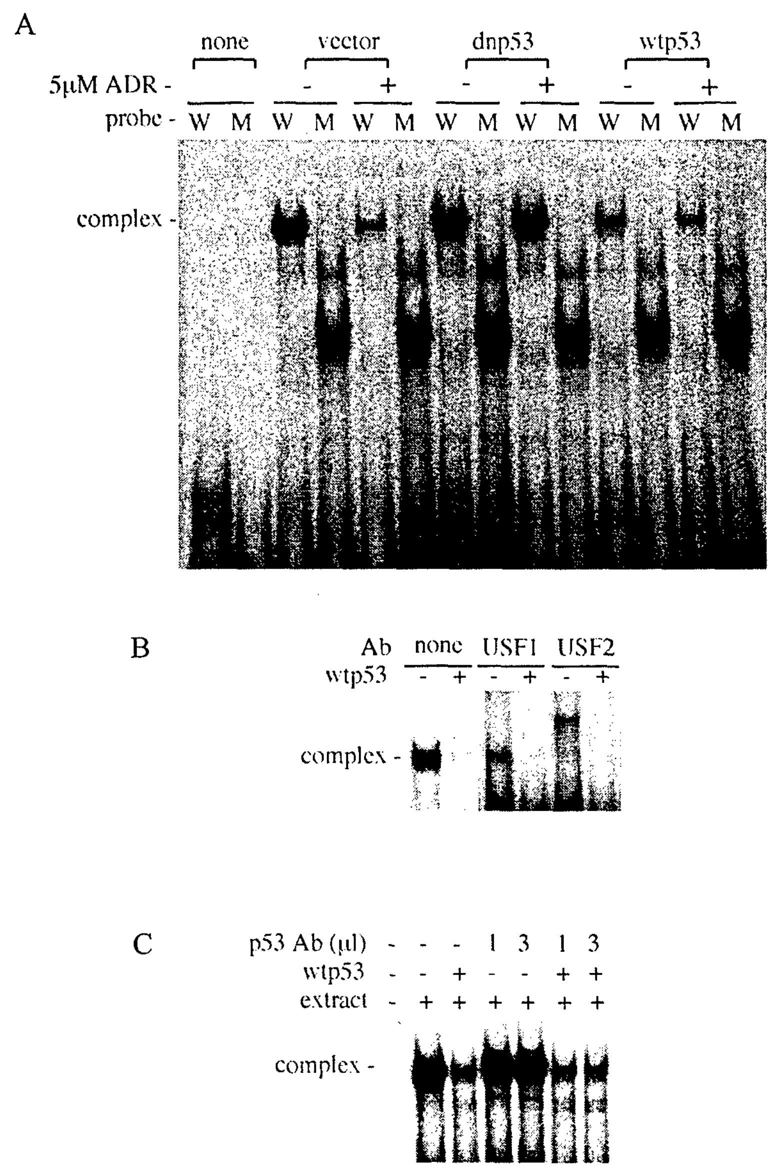
Wu, et al. Figure 1



A
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative luciferase activity
5μM ADR (-)
5μM ADR (+)
vector
wtp53
B
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative luciferase activity
5μM ADR (-)
5μM ADR (+)
vector
wtp53
dnp53
C
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative luciferase activity
0
25
125
250
500
wtp53 (ng)
D
U20S
Saos-2
Relative luciferase activity
0.0 2.5 5.0 10
ADR (μM)
0.0 2.5 5.0 10
ADR (μM)
E
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative luciferase activity
MCF7/CMV
MCF7/E6
0.0 1.0 5.0
ADR (μM)
0.0 1.0 5.0
ADR (μM)

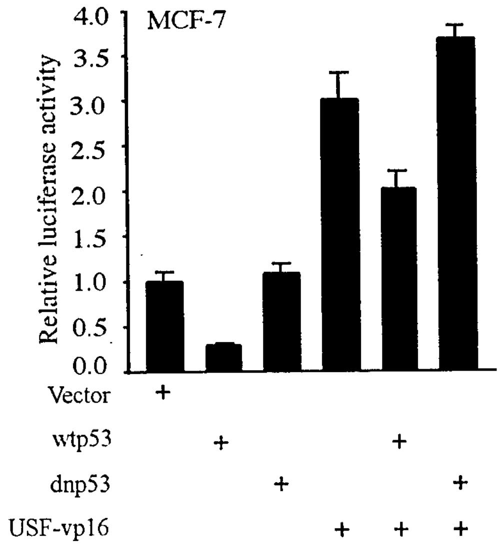


Wu, et al. Figure 3



Wu, et al. Figure 4

A



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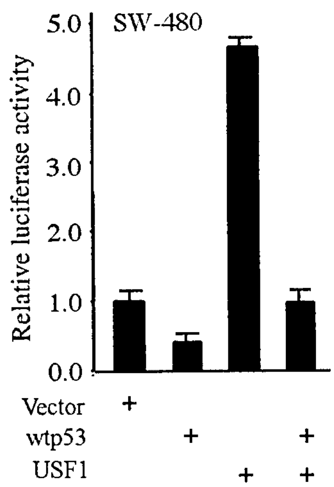

Wu, et al. Figure 5

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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